



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A01N 1/02, 43/04, 63/00, 65/00, G01N 33/53, A61K 31/70, 38/00, 39/00, 35/14		A1	(11) International Publication Number: WO 97/34473
			(43) International Publication Date: 25 September 1997 (25.09.97)
(21) International Application Number: PCT/US97/05076 (22) International Filing Date: 21 March 1997 (21.03.97) (30) Priority Data: 60/013,820 21 March 1996 (21.03.96) US 60/016,626 1 May 1996 (01.05.96) US 60/016,659 1 May 1996 (01.05.96) US 60/026,584 18 September 1996 (18.09.96) US (60) Parent Applications or Grants (63) Related by Continuation US 60/013,820 (CIP) Filed on 21 March 1996 (21.03.96) US 60/016,626 (CIP) Filed on 1 May 1996 (01.05.96) US 60/016,659 (CIP) Filed on 1 May 1996 (01.05.96) US 60/026,584 (CIP) Filed on 18 September 1996 (18.09.96) (71) Applicant (for all designated States except US): THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; West 116th Street and Broadway, New York, NY 10027 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only): LEDERMAN, Seth [US/US]; Apartment 8C, 533 West 112th Street, New York, NY 10032 (US). FRANK, Dale, M. [US/US]; Apartment 4, 261 West 11th Street, New York, NY 10014 (US). CLEARY, Aileen, M. [US/US]; 60 Haven Avenue, New York, NY 10032 (US). (74) Agent: WHITE, John, P.; Cooper & Dunham L.L.P., 1185 Avenue of the Americas, New York, NY 10036 (US). (81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.	
(54) Title: CRAF1 (TRAF-3) ISOFORMS AND USES THEREOF			
(57) Abstract			
<p>The present invention provides an isolated CRAF1 peptide encoded by the nucleic acid sequence shown from base 169 to base 2381 of Figures 1A-1P or a variant thereof. One embodiment of the present invention is an isoform of the CRAF1 peptide. The present invention also provides for a method of inhibiting activation by CD40 ligand of cells expressing CD40 on the cell surface, comprising providing the cells with the CRAF1 peptide or variant thereof, the peptide being present in an amount effective to inhibit activation of the cells. The present invention further provides for a method of treating a condition characterized by an aberrant or unwanted level of CD40-mediated intracellular signalling, in a subject, comprising providing the subject with a therapeutically effective amount of a peptide of this invention capable of inhibiting CD40-mediated intracellular signalling in cells bearing CD40 on the cell surface.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

CRAF1 (TRAF-3) ISOFORMS AND USES THEREOF

5 This application claims the benefit of U.S. Provisional
No. 60/013,820, filed March 21, 1996; U.S. Provisional
No. 60/026,584, filed September 18, 1996; U.S.
Provisional No. 60/016,659, filed May 1, 1996; and U.S.
Provisional No. 60/016,626, filed May 1, 1996, the
10 contents of which are hereby incorporated by reference
into the present application.

The invention disclosed herein was made with Government
support under NIH Grant No. RO1-CA55713 from the
15 Department of Health and Human Services. Accordingly,
the U.S. Government has certain rights in this
invention.

Throughout this application, various references are
20 referred to in the text within parentheses in full or
within parentheses by number. Disclosures of these
publications in their entireties are hereby incorporated
by reference into this application to more fully
describe the state of the art to which this invention
25 pertains. Full bibliographic citation for those
references referred to by number may be found at the end
of this application, preceding the claims.

The following standard abbreviations are used throughout
30 to refer to amino acids:

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
35 F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
40 L	Leu	Leucine	Y	Tyr	Tyrosine

Background of the Invention

CD40 (1) is a receptor on B cells that interacts with the helper T cell surface protein CD40L (CD40 ligand, also known as T-BAM, gp39, or TRAP) (2-4). CD40L is found particularly on lymphoid follicle CD4⁺ T lymphocytes, where it delivers a contact-dependent signal that stimulates B cell survival, growth, and differentiation (2-4). Signaling through CD40 rescues B cells from apoptosis induced by Fas (CD95) or by cross-linking of the immunoglobulin M (IgM) complex (5); it also induces B cells to differentiate and to undergo Ig isotype switching (3) and to express CD80 (B7 or BB-1) (6). The crucial role of CD40L-CD40 interaction is illustrated by humans with defects in CD40L, who manifest a serious immune deficiency syndrome, the X-linked hyper-IgM syndrome (HIGMX-1) characterized by an absence of IgG, IgA, and IgE, elevated IgM, and no lymphoid follicles (7). The essential roles of CD40L and CD40 in the phenotype of HIGMX-1 syndrome has been confirmed by targeted disruption of either CD40L (8) or CD40 (9) in mice. In addition to B cells, CD40 is also expressed by follicular dendritic cells (10), dendritic cells (11), activated macrophages (12), epithelial cells (including thymic epithelium) (13), and a variety of tumor cells.

Stimulation of CD40 causes the tyrosine phosphorylation of multiple substrates including Src family kinases such as p53-p56^{l^{yn}}, activates multiple serine-threonine-specific protein kinases, and induces the phosphorylation of phospholipase C- γ 2 and of phosphoinositol-3' kinase (14). CD40 ligation also stimulates protein kinase C-independent activation of the mitogen-activated protein kinases (MAPK) family, including the extracellular signal-regulated protein kinases 1 and 2 (ERK) and the c-Jun NH2-terminal kinases

- 3 -

(JNK isoforms) (Li, Y. et al. (1996) J. Immunol. 157:1440). In mice the CD40 cytoplasmic tail is necessary for signaling (15). Proteins which interact with the cytoplasmic tail of CD40 have been described (H.M. Hu, et al., J. Biol. Chem. 269: 30069 (1994); and G. Mosialos, et al., Cell 80:389 (1995)). These proteins are the same as CRAF1.

CD40 signaling is known to be complex and may be mediated or modulated by TRAF-2 (Rothe, M. et al. (1995) Science 269:1424), TRAF-5 (Nakano, H. (1996) J. Biol. Chem. 271:14661) and TANK (Cheng, G. And Baltimore, D. (1996) Genes Dev. 10:963; Rothe, M. et al. (1996) PNAS USA 93:8241) either alone or in interactions with TRAF-3. TRAF-2 signals may be modulated by TANK (or I-TRAF) (Rothe, M. et al. (1996) PNAS USA 93:8241) or by A20, a TNFa-induced gene product (Song, H.Y. et al. (1996) PNAS USA 93:6721).

Summary of the Invention

5 The present invention provides an isolated CRAF1 peptide encoded by the nucleic acid sequence shown from base 169 to base 2381 of Figures 1A-1P or a variant thereof. One embodiment of the present invention is an isoform of the CRAF1 peptide. The present invention also provides for a method of inhibiting activation by CD40 ligand of
10 cells expressing CD40 on the cell surface, comprising providing the cells with the CRAF1 peptide or variant thereof, the peptide being present in an amount effective to inhibit activation of the cells. The present invention further provides for a method of
15 treating a condition characterized by an aberrant or unwanted level of CD40-mediated intracellular signaling, in a subject, comprising providing the subject with a therapeutically effective amount of a peptide of this invention capable of inhibiting CD40-mediated
20 intracellular signaling in cells bearing CD40 on the cell surface.

Description of the Figures

Figures 1A-1P. Sequence of TRAF-3 (CRAF-1) and Isoforms thereof. Sequence data of CRAF1 is shown including cDNA sequence of isoforms of CRAF1 including CRAF1-a (p55; p60 isoform), CRAF1-b (p70; p77 isoform), smaller CRAF1 pre-peptides (p5 isoform and p15 isoform), and other deletion isoforms and alternative splice possibilities. The DNA and amino acid sequences of such isoforms are also indicated. The base pair number and the amino acid residue number are indicated.

Figures 2A-2B. Fig. 2A. Schematic diagram of the genomic structure of the zinc finger region of CRAF-1. Fig 2B. Schematic diagram of the cDNA structure of the zinc finger regions of CRAF-1, CRAF-1 (del aa 218-242), and CRAF-1 (del aa 191-242), as indicated. Predicted amino acid sequences of human CRAF1-a (residues 1-568).

Figure 3. Exon Organization, Numbering and Arrangement in TRAF-3 cDNAs.

Figure 4. Predicted CRAF1 zinc fingers, corresponding to residues 110 to 264. The zinc fingers are numbered consecutively from 1 to 5, proceeding from the amino terminus to the carboxy terminus. The numbering of the amino acids is based on the sequence shown in Figures 1A-1P, in which the initial "M" residue is +1.

Figure 5. Differential expression of CRAF1 isoforms in hyper-IgM patients. Western blot analysis. Cell lysates from 1×10^7 cells of B cell tumor lines (lanes 1-7 non-transformed, 8-12 EBV-transformed) were immunoprecipitated with a rabbit antiserum directed against TRAF-3 (CRAF-1) molecule. Following the SDS-PAGE, the proteins were transferred to a nitrocellulose sheet and probed with the anti-CRAF-1 antiserum. The

- 6 -

specificity of this antiserum is demonstrated by the fact that the low molecular weight CRAF-1, p60 CRAF, (lanes 1-4, 6-10, and 12) can be specifically inhibited by a synthetic peptide (corresponding to the N-terminal 22 amino acid residues of CRAF-1) which was used as the immunogen to generate the rabbit anti-CRAF-1 antiserum (lane 5). The 55 kDa component found in every lane is the immunoglobulin heavy chain (IgH-chain). BJAB (tx CD40) is BJAB transfected with CD40 and overexpressing CD40. RCC/CRAF is Ramos CC transfected with and stably expressing a cDNA construct encoding CRAF1-a (TRAF-3-p70), and overexpressing CRAF1-a mRNA. BL41 is a B cell tumor cell line.

The expression of the p60 CRAF-1 is comparable in all the cell lines examined except the EBV-transformed B cells from patient B, in which only trace amounts of the p60 CRAF1 was detected. This result shows that p60 CRAF1 is normally expressed in B lymphocytes.

A high molecular weight CRAF1, p70 CRAF (TRAF-3-p70), was identified in EBV-transformed B cells (lanes 9-12), but not in non-EBV-transformed cell lines. However the expression level of p70 CRAF1 varies. In EBV-transformed B cells established from normal B lymphocytes, p70 CRAF is only expressed at low levels (lane 9). However, in EBV-transformed B cells from patient A, the expression of p60 CRAF and p70 CRAF1 is comparable (lane 10). Moreover, in EBV-transformed B cells from patient B, the expression of p70 CRAF1 is not only dominant but also dramatically up-regulated and only trace amounts, of the p60 CRAF1 was detected in these cells (lane 11). In contrast to the hyper IgM patients A and B (which are known to have normal CD40L) in EBV-transformed cells established from patient C, the p70 CRAF1 expression is below the level of detection (lane 12).

- 7 -

Figure 6. Genomic localization and Orientation of the human TRAF-3 Gene on 14q32.

5 **Figures 7A-7B.** The Entire TRAF-3 Gene is Encoded on Two pAC clones. A schematic of two pAC clones is illustrated along with peptide-encoding regions.

10 **Figure 8.** Complexity of CD40/p80/LT- β R Interactions with TRAF-3, TRAF-1 and TRAF-2.

Figures 9A-9C. Relationship of CRAF1/TRAF-3 Isoforms to Truncated C26 clone and peptide domains.

15 **Figures 10A-10D.** 5'-Untranslated Region of Human TRAF-3 Gene with Transcription Regulatory Functions

Detailed Description

This invention provides for an isolated CRAF1 peptide encoded by the nucleic acid sequence shown from base 169
5 to base 2381 of Figures 1A-1P or a variant thereof.

The CRAF1 peptide may be an isoform of the CRAF1 peptide. The isoform may comprise a p5 peptide, a p15 peptide, a p55del9 peptide, a p70del9 peptide, a
10 p55del9,10 peptide, a p70del9,10 peptide, a p55del-8,9 peptide, a p70del-8,9 peptide as shown in Figures 1A-1P.

The CRAF1 peptide may comprise from zero to four zinc finger domains; wherein when the isoform comprises zinc
15 finger domain 1, 2 and 5, the peptide further comprises one or both of zinc finger domains 3 and 4. The isoform may comprise a CRAF1 peptide or variant thereof comprising zero, one, two or four zinc finger domains. The peptide may have deleted zinc finger domains 2, 3
20 and 4. The peptide may have deleted amino acids 191 to 242 or amino acids 313 to 364 as shown in Figures 1A-1P.

The peptide may comprise the sequence
GARRGRRVREPGLQPSRDFPAGGSRGGRRLLFPAPRHGAARGA(E/K) (R/C) CG
25 PRR(Q/R) TRPAPLSRPSGDGP(Q/R) ELLFPK (Seq I.D. No. ____) or a variant thereof capable of inhibiting CD40-mediated cell activation.

The isoform may have a molecular weight of about 5 kDa
30 or about 15 kDa.

As used herein "CRAF1" is also termed "TRAF-3". CRAF1 nucleic acid encompasses the nucleic acid sequence shown in Figures 1A-1P. "CRAF1-a" is also termed "TRAF-3-p55"
35 or "p55" or "CRAF1(p55)" or "TRAF-3 (p55)" or "CRAF1(p60)". "CRAF1-b" is also termed "TRAF-3-p70" or "p70" or "CRAF1(p70)" or "TRAF-3 (p 70)". p60 is also termed p55

- 9 -

(p60 = p55) and p77 is also termed p70 (p70 = p77). "P70-i" as used herein encompasses all of the isoforms of p70. and p70-i is represented in Figures 1A-1P as the longest isoform of p70.

5

As used herein, "CRAF1 gene" encompasses a nucleic acid sequence comprising a genomic sequence of a CRAF1 nucleic acid. Two embodiments of a CRAF1 gene are the sequences contained in the two pAC clone nucleic acid constructs (pAC clone number 34 and pAC clone number 10 167) which have been deposited on March 21, 1997 with the American Type Tissue Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of 15 Microorganism for the Purposes of Patent Procedure. pAC clone no. 34 was accorded ATCC Accession No. 97963 and pAC clone no. 167 (P1 artificial chromosome 167) was accorded ATCC Accession No. 97962. Another 20 embodiment of a CRAF1 gene is the human CRAF1 gene. A portion of the human CRAF1 gene is shown in Figures 1A-1P from nucleotide numbers 1-2918. Other embodiments of the present invention provide for the homologous murine CRAF1 gene, and the analogous homolog CRAF1 gene in 25 other species of animals. Further embodiments of a CRAF1 gene are the sequences contained in the following deposits: pE, pGM and pZAC which have been deposited on March 21, 1997 with the American Type Tissue Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, 30 Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. pE was accorded ATCC Accession No. 97965 and pMG was was accorded ATCC Accession No. 97964 and pZAC 35 was accorded ATCC Accession No. 97966.

- 10 -

As used herein, "CRAF1 nucleic acid" encompasses the DNA nucleic acid sequence shown in **Figures 1A-1P** from (169-2381) which encodes CRAF1 (p70) or p70-i as designated in **Figures 1A-1P**. One embodiment of the present invention is CRAF1 nucleic acid comprising DNA, recombinant DNA, cDNA, mRNA or RNA. Another embodiment of the present invention is the reverse complement of CRAF1 nucleic acid. A further embodiment of the present invention is a nucleic acid molecule comprising at least a portion of the CRAF1 nucleic acid sequence shown in **Figures 1A-1P** in an antisense orientation. The nucleic acid may be an isolated nucleic acid or a purified nucleic acid which nucleic acid is separated from cellular particles substantially. Such isolation or purification would be known to one of skill in the art, see Sambrook, et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989).

One embodiment of this invention is a nucleic acid comprising of a portion of the human CRAF1 nucleic acid sequence shown in **Figures 1A-1P**. Such embodiments may be isoforms (p70-i) of the CRAF1 nucleic acid such as deletion mutants, insertion mutants or substitution mutants, wherein portions of the nucleic acid sequence is deleted, inserted or substituted, respectively. Examples of such isoforms are indicated in **Figures 1A-1P**, i.e. p5, p15, p55-1, etc. Further embodiments of this invention are described in **Tables 1 and 2** (see Experimental Details section hereinbelow). Another embodiment of the present invention is the deposited cDNA clone IIIb, ATCC No. 97489. A further embodiment of the present invention are pAC clones which encompass the entire CRAF1 genomic sequence or a portion of the CRAF1 genomic sequence.

This invention provides for a nucleic acid comprising

- 11 -

the cDNA nucleotide sequence of human CRAF1 IIIb clone (TRAF-3(p55)) which was deposited in GenBank with accession number U21092. This plasmid, pBluescriptSKII+/IIIb, was deposited on March 21, 1996 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid pBluescriptSKII+/IIIb was accorded ATCC Accession Number 97489.

This invention provides for alleles of the CRAF1 nucleic acid. This invention provides for human CRAF1 nucleic acid (as shown in **Figures 1A-1P**) and also provides for the homologous murine CRAF1 nucleic acid, as well as the homologous CRAF1 nucleic acids in other species of animals.

The present invention also provides for upstream and downstream regulatory nucleic acid sequences of CRAF1 nucleic acid. One embodiment of this invention is shown in **Figures 1A-1P** from nucleotides 1-168. Another embodiment of the present invention is shown in **Figures 1A-1P** from nucleotide 2382-2918. Another embodiment of the invention is shown in **Figures 2A-2B** which shows the 5' untranslated region of human TRAF-3 gene (CRAF1 gene) with has been shown to function as a transcriptional regulator or modulator. This upstream sequence capable of being activated to increase expression of a gene (e.g. transactivated) by EBV infection. This sequence and variants thereof may be useful the modulation and/or expression of heterologous genes as part of recombinant, heterologous nucleic acid constructs. Such constructs may be used in gene therapy as described in more detail hereinbelow.

- 12 -

The present invention also provides for nucleic acid probes derived from the nucleic acid sequence as shown in Figures 1A-1P and in Tables 1-4. Such probes may be useful in diagnostic testing of subjects for disease states and for determination of levels of CRAF1 peptide expression or lack thereof. Such probes may have other uses such as a monitor use in gene therapy or alternative therapy of subjects suffering from a CRAF1 related disorder.

The present invention also provides for a CRAF1 nucleic acid molecule linked to a vector. The vector may be a self-replicating vector or a replicative incompetent vector. The vector may be a pharmaceutically acceptable vector for methods of gene therapy. An example of replication incompetent vector is LNL6 (Miller, A.D. et al. (1989) BioTechniques 7:980-990).

The present invention provides for CRAF1 nucleic acid which is produced by polymerase chain reaction (PCR). An isolated CRAF1 nucleic acid may be isolated by using PCR. Such reactions are well known to one of skill in the art. [U.S. Patent Nos. 4,754,065; 4,800,159;; 4,683,195 and 4,683,202 provide PCR techniques and methods and these U.S. Patents are hereby incorporated by reference in their entirety.]

As used herein, "CRAF1 peptide" encompasses the amino acid sequence shown in **Figures 1A-1P** encoded by the nucleotide sequence from base 169 to base 2831 (therein termed p70-i). CRAF1 peptide is also termed TRAF-3 peptide (TRAF-3-p55 or CRAF1(p70)).

This invention encompasses isoforms of the CRAF1 peptide, the sequence of several of which are shown in Figures 1A-1P, i.e. p5, p15, p55-i, p55del-9, etc. Such embodiments and further embodiments of isoforms of the

- 13 -

CRAF1 peptide are described in Tables 1 and 2. Embodiments of CRAF1 peptide include peptides produced from alternatively spliced transcripts derived from a CRAF1 nucleic acid. CRAF1 peptide isoforms with reduced numbers of zinc finger regions, truncated CRAF1 peptide. Another embodiment of the present invention is a prematurely terminated CRAF1 peptide, e.g. p5 and p15 as shown in Figures 1A-1P and on Tables 1 and 2.

In another embodiment of the present invention CRAF1 nucleic acid may also be a synthetic nucleic acid or a mimetic of a nucleic acid which may have increased bioavailability, stability, potency or decreased toxicity. Such synthetic nucleic acids may have alterations of the basic A, T, C or G or U bases or sugars which make up the nucleotide polymer to as to alter the effect of the nucleic acid.

This invention provides an isolated protein comprising a CRAF1 peptide (TRAF-3-p70) domain which comprises GARRGRRVREPGGLQPSRDFPAGGSRGGRRLLFPAPRHGAARGA (E/K) (R/C) CG PRR (Q/R) TRPAPLSRPSGDGP (Q/R) ELLFPK (Seq I.D. No. ____), or a variant thereof capable of inhibiting CD40-mediated cell activation (Figures 1A-1P). The amino acids in parentheses are alternatives; one amino acid was found to be coded for in the cloned cDNA and the other amino acid was found to be encoded by the genomic sequence. Thus, it is likely that there are polymorphisms at these sites. This is a 71 amino acid sequence encoded by sequence which is found at the amino-terminal domain of CRAF1 nucleic acid as shown in Figures 1A-1P.

In an embodiment of this invention the protein further comprises CRAF1-a (TRAF-3-p55 or CRAF1(p55)) or a variant thereof adjacent to the carboxy-terminus of the CRAF1-b (TRAF-3-p70 or CRAF1(p55)) domain. In an embodiment the molecular weight of the CRAF1 peptide is

- 14 -

about 70 kDa. In another embodiment, an isoform of the CRAF1 peptide has a molecular weight of about 5 kDa or about 15 kDa. In an embodiment the CRAF1-b (p70) amino terminal domain comprises at least about 71 amino acids.

5 In another embodiment, it comprises from about 120 to about 150 amino acids. Preferably, it comprises 122 amino acids.

10 This invention provides a CRAF1 peptide having a molecular weight of about 70 kDa. In an embodiment, the CRAF1 peptide comprises an amino acid sequence encoded by exons 1-13. In more specific embodiments, the CRAF1 peptide comprises an amino acid sequence that is encoded by the exons as shown in the examples in Tables 1 and 2.

15 As used herein, "variants" encompass the following: Variants can differ from naturally occurring CRAF1 peptide (TRAF-3 peptide) in amino acid sequence or in ways that do not involve sequence, or both. Variants in amino acid sequence are produced when one or more amino acids are substituted with a different natural amino acid, an amino acid derivative or non-native amino acid. When a nucleic acid molecule encoding the protein is expressed in a cell, one naturally occurring amino acid

20 will generally be substituted for another. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics such as substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar

25 neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine,

30

35

- 15 -

lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

5 Other conservative substitutions can be taken from Table A, and yet others are described by Dayhoff in the Atlas of Protein Sequence and Structure (1988).

Table A: Conservative Amino Acid Replacements

For Amino Acid	Code	Replace with any of
10 Alanine	A	D-Ala, Gly, beta-ALA, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
15 Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, Beta-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Met, D-Met
20 Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val, Norleu

- 16 -

Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans 3,4 or 5-phenylproline, cis 3,4 or 5 phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O) D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Other variants within the invention are those with modifications which increase peptide stability. Such variants may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are: variants that include residues other than naturally occurring L-amino acids, such as D-amino acids or non-naturally occurring or synthetic amino acids such as beta or gamma amino acids and cyclic variants. Incorporation of D- instead of L-amino acids into the polypeptide may increase its resistance to proteases. See, e.g., U.S. Patent No. 5,219,990.

The protein of this invention may also be modified by various changes such as insertions, deletions and substitutions, either conservative or nonconservative where such changes might provide for certain advantages in their use.

- 17 -

In other embodiments, variants with amino acid substitutions which are less conservative may also result in desired derivatives, e.g., by causing changes in charge, conformation and other biological properties. Such substitutions would include for example, substitution of hydrophilic residue for a hydrophobic residue, substitution of a cysteine or proline for another residue, substitution of a residue having a small side chain for a residue having a bulky side chain or substitution of a residue having a net positive charge for a residue having a net negative charge. When the result of a given substitution cannot be predicted with certainty, the derivatives may be readily assayed according to the methods disclosed herein to determine the presence or absence of the desired characteristics.

Variants within the scope of the invention include proteins and peptides with amino acid sequences having at least eighty percent homology with the CRAF1 upstream protein sequence, or CRAF1-b. More preferably the sequence homology is at least ninety percent, or at least ninety-five percent.

Just as it is possible to replace substituents of the scaffold, it is also possible to substitute functional groups which decorate the scaffold with groups characterized by similar features. These substitutions will initially be conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. Non-sequence modifications may include, for example, in vivo or in vitro chemical derivatization of portions of the protein of this invention, as well as changes in acetylation, methylation, phosphorylation, carboxylation or glycosylation.

In a further embodiment the protein is modified by

- 18 -

chemical modifications in which activity is preserved. For example, the proteins may be amidated, sulfated, singly or multiply halogenated, alkylated, carboxylated, or phosphorylated. The protein may also be singly or multiply acylated, such as with an acetyl group, with a farnesyl moiety, or with a fatty acid, which may be saturated, monounsaturated or polyunsaturated. The fatty acid may also be singly or multiply fluorinated. The invention also includes methionine analogs of the protein, for example the methionine sulfone and methionine sulfoxide analogs. The invention also includes salts of the proteins, such as ammonium salts, including alkyl or aryl ammonium salts, sulfate, hydrogen sulfate, phosphate, hydrogen phosphate, dihydrogen phosphate, thiosulfate, carbonate, bicarbonate, benzoate, sulfonate, thiosulfonate, mesylate, ethyl sulfonate and benzensulfonate salts.

This invention provides a method of inhibiting activation by CD40 ligand of cells bearing CD40 on the cell surface, comprising providing the cells with the protein of this invention, the protein being present in an amount effective to inhibit activation of the cells.

The present invention further provides a method of inhibiting activation by CD40 ligand of cells expressing CD40 on the cell surface, comprising providing the cells with the peptide of the invention, the peptide being present in an amount effective to inhibit activation of the cells.

In one embodiment, the cells are provided with the peptide by introducing into the cells a nucleic acid sequence encoding the peptide under conditions such that the cells express an amount of the peptide effective to inhibit activation of the cells.

- 19 -

In another embodiment, the nucleic acid sequence is operably linked to a transcriptional control sequence recognized by the cell. The nucleic acid sequence may be a plasmid. The CD40-bearing cells may be selected from the group consisting of B cells, fibroblasts, endothelial cells, epithelial cells, T cells, basophils, macrophages, Reed-Steinberg cells, dendritic cells, renal cells, and smooth muscle cells. The B cells may be resting B cells, primed B cells, myeloma cells, lymphocytic leukemia B cells, or B lymphoma cells. The epithelial cells may be keratinocytes. The fibroblasts may be synovial membrane fibroblasts, dermal fibroblasts, pulmonary fibroblasts, or liver fibroblasts.

The renal cells may be selected from the group consisting of glomerular endothelial cells, mesangial cells, distal tubule cells, proximal tubule cells, parietal epithelial cells, visceral epithelial cells, cells of a Henle limb, and interstitial inflammatory cells.

The parietal epithelial cells may be crescent parietal epithelial cells.

The smooth muscle cells may be smooth muscle cells of the bladder, vascular smooth muscle cells, aortic smooth muscle cells, coronary smooth muscle cells, pulmonary smooth muscle cells, or gastrointestinal smooth muscle cells.

The gastrointestinal smooth muscle cells may be esophageal smooth muscle cells, stomachic smooth muscle cells, smooth muscle cells of the small intestine, or smooth muscle cells of the large intestine.

The invention provides a method of providing a subject

- 20 -

with an amount of a CRAF 1 peptide effective to inhibit activation by CD40 ligand of cells bearing CD40 on the cell surface in the subject, comprising: introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding the CRAF1 peptide, under conditions such that the cells express in the subject an activation inhibiting effective amount of the peptide.

The introducing of the nucleic acid into cells of the subject may comprise a) treating cells of the subject ex vivo to insert the nucleic acid sequence into the cells; and b) introducing the cells from step a) into the subject.

The subject may be a mammal. The mammalian subject may be a human.

An embodiment of the present invention is a method of treating a condition characterized by an aberrant or unwanted level of CD40-mediated intracellular signaling, in a subject, comprising providing the subject with a therapeutically effective amount of a CRAF1 peptide capable of inhibiting CD40-mediated intracellular signaling in cells bearing CD40 on the cell surface.

The peptide may be provided by introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding the peptide under conditions such that the cells express the peptide according to this method.

The condition may be organ rejection in a subject receiving transplant organs, or an immune response in a subject receiving gene therapy. The transplant organ may be a kidney, heart or liver. The condition may be a CD40-dependent immune response. The CD40-dependent immune response may be an autoimmune response in a subject suffering from an autoimmune disease.

- 21 -

The autoimmune disease may comprise rheumatoid arthritis, Myasthenia gravis, systemic lupus erythematosus, Graves' disease, idiopathic thrombocytopenia purpura, hemolytic anemia, diabetes mellitus, a drug-induced autoimmune disease, psoriasis, or hyper IgE syndrome.

The drug-induced autoimmune disease may be drug-induced lupus. The immune response may comprise induction of CD23, CD80 upregulation, rescue from CD95-mediated apoptosis, rescue from apoptosis in a subject undergoing chemotherapy against a tumor, or autoimmune manifestations of an infectious disease.

The autoimmune manifestations may be derived from Reiter's syndrome, spondyloarthritis, Lyme disease, HIV infections, syphilis or tuberculosis. The condition may be an allergic response. The allergic response may be hay fever or a penicillin allergy. The condition may be dependent on CD40 ligand-induced activation of fibroblast cells. The condition may be selected from the group consisting of arthritis, scleroderma, and fibrosis. The arthritis may be rheumatoid arthritis, non-rheumatoid inflammatory arthritis, arthritis associated with Lyme disease, or osteoarthritis. The fibrosis may be pulmonary fibrosis, hypersensitivity pulmonary fibrosis, or a pneumoconiosis. The pulmonary fibrosis may be pulmonary fibrosis secondary to adult respiratory distress syndrome, drug-induced pulmonary fibrosis, idiopathic pulmonary fibrosis, or hypersensitivity pneumonitis. The pneumoconiosis may be asbestosis, siliconosis, or Farmer's lung. The fibrosis may be a fibrotic disease of the liver or lung. The fibrotic disease of the lung may be caused by rheumatoid arthritis or scleroderma. The fibrotic disease of the liver may be selected from the group consisting of: Hepatitis-C; Hepatitis-B; cirrhosis; cirrhosis of the

- 22 -

liver secondary to a toxic insult; cirrhosis of the liver secondary to drugs; cirrhosis of the liver secondary to a viral infection; and cirrhosis of the liver secondary to an autoimmune disease. The toxic
5 insult may be alcohol consumption. The viral infection may be Hepatitis B, Hepatitis C, or hepatitis non-B non-C. The autoimmune disease may be primary biliary cirrhosis, or Lupoid hepatitis. The condition may be dependent on CD40 ligand-induced activation of
10 endothelial cells. The condition may be selected from the group consisting of atherosclerosis, reperfusion injury, allograft rejection, organ rejection, and chronic inflammatory autoimmune diseases.

15 The atherosclerosis may be accelerated atherosclerosis associated with organ transplantation.

The chronic inflammatory autoimmune disease may be vasculitis, rheumatoid arthritis, scleroderma, or
20 multiple sclerosis.

The condition may be dependent on CD40 ligand-induced activation of epithelial cells.

25 In one embodiment of the invention, the epithelial cells are keratinocytes, and the condition is psoriasis.

The condition may be an inflammatory kidney disease. The inflammatory kidney disease may not be initiated by
30 autoantibody deposition in kidney. The kidney disease may be selected from the group consisting of: membranous glomerulonephritis; minimal change disease/acute tubular necrosis; pauci-immune glomerulonephritis; focal segmental glomerulosclerosis; interstitial nephritis; antitissue
35 antibody-induced glomerular injury; circulating immune-complex disease; a glomerulopathy associated with a multisystem disease; and drug-induced glomerular

- 23 -

disease.

The antitissue antibody-induced glomerular injury may be anti-basement membrane antibody disease.

5

The circulating immune-complex disease may be selected from the group consisting of: infective endocarditis; leprosy; syphilis; hepatitis B; malaria; and a disease associated with an endogenous antigen.

10

The endogenous antigen may be DNA, thyroglobulin, an autologous immunoglobulin, erythrocyte stroma, a renal tubule antigen, a tumor-specific antigen, or a tumor-associated antigen.

15

The glomerulopathy may be associated with a multisystem disease is selected from the group consisting of: diabetic nephropathy; systemic lupus erythematosus; Goodpasture's disease; Henoch-Schönlein purpura; polyarteritis; Wegener's granulomatosis; cryoimmunoglobulinemia; multiple myeloma; Waldenström's macroglobulinemia; and amyloidosis.

20

25

The pauci-immune glomerulonephritis may be ANCA+ pauci-immune glomerulonephritis, or Wegener's granulomatosis. The interstitial nephritis may be drug-induced interstitial nephritis. The kidney disease may affect renal tubules. The kidney disease which affects renal tubules may be selected from the group consisting of: a kidney disease associated with a toxin; a neoplasia; hypersensitivity nephropathy; Sjögren's syndrome; and AIDS.

30

35

The condition may be a smooth muscle cell-dependent disease. The smooth muscle cell-dependent disease may be a vascular disease. The vascular disease may be atherosclerosis. The smooth muscle cell-dependent

- 24 -

disease may be a gastrointestinal disease. The gastrointestinal disease may be selected from the group consisting of esophageal dysmotility, inflammatory bowel disease, and scleroderma. The smooth muscle cell-dependent disease may be a bladder disease.

The condition may be associated with Epstein-Barr virus. The condition may be selected from the group consisting of mononucleosis, B cell tumors, Burkitt's lymphoma, and nasopharyngeal carcinoma. The treatment may not increase susceptibility of the subject to pneumocystis pneumonia, atypical infections, or tumors.

The present invention provides for an isolated nucleic acid molecule encoding a CRAF1 peptide. The nucleic acid molecule may be DNA, RNA, cDNA, recombinant DNA, or mRNA.

One embodiment of the invention is a vector comprising the CRAF1 nucleic acid molecule or variants or isoforms thereof operably linked to a transcriptional control sequence recognized by a host cell transformed with the vector. The vector may be a plasmid.

The present invention provides a method of differentiating a subject with hyper-IgM syndrome due to an abnormal CD40 receptor-associated factor polypeptide from those with hyper-IgM syndrome due to other causes, comprising: detecting in an extract from a cell derived from the subject, the presence of a nucleic acid encoding the abnormal CD40 receptor-associated factor, thereby differentiating the subject with hyper-IgM syndrome due to an abnormal CD40 receptor-associated factor polypeptide from those with hyper-IgM syndrome due to other causes.

The abnormal CD40 receptor-associated factor polypeptide

- 25 -

may be abnormal CD40 receptor-associated factor 1. The subject may be heterozygous for the abnormal CD40 receptor-associated factor. The subject may express CD40 ligand normally. The subject may be a human. The cell
5 may be a B cell. The cell may be derived from a cell culture. The cell may be derived from a bodily fluid. The nucleic acid may encode a truncated CD40 receptor-associated factor polypeptide. The nucleic acid may encode the CD40 receptor-associated factor
10 truncated at the carboxy terminus. The CD40 receptor-associated factor polypeptide may be truncated by at least about 170 amino acid residues. The CD40 receptor-associated factor polypeptide may be truncated by at least about 244 amino acid residues. The nucleic acid
15 may encode the CD40 receptor-associated factor truncated at the amino terminus. The CD40 receptor-associated factor polypeptide may be truncated by at least about 100 amino acid residues. The nucleic acid in the extract may be mRNA, DNA.

20 The method may further comprise amplifying the nucleic acid prior to detecting, and wherein the detecting is detecting of the amplified nucleic acid.

25 The detecting may comprise: contacting the nucleic acid to be detected with a probe, wherein, if the nucleic acid to be detected is DNA, the probe is capable of hybridizing to a coding or noncoding strand of a unique sequence encoding a normal CD40 receptor-associated
30 factor; and if the nucleic acid to be detected is RNA, the probe is capable of hybridizing to a unique sequence encoding a normal CD40 receptor-associated factor, under stringent conditions which would permit hybridization with the unique sequence if present; and detecting the
35 absence of a hybrid of the probe and the nucleic acid to be detected, thereby detecting the nucleic acid encoding the abnormal CD40 receptor-associated factor. The probe

- 26 -

may comprise at least nine nucleotides, at least twelve nucleotides, or at least fifteen nucleotides. The probe may be labeled. The label may be a radioactive isotope. The label may be iodine-125. The determining may comprise sequencing the nucleic acid.

The present invention provides for a method of providing a subject with an immunosuppressant effective amount of an abnormal CD40 receptor-associated factor, comprising: introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding an abnormal CD40 receptor-associated factor polypeptide, under conditions such that the cells express in the subject an immunosuppressant effective amount of the abnormal CD40 receptor-associated factor.

One embodiment of the present invention is an antibody or portion thereof capable of specifically binding to a CD40 receptor-associated factor. The CD40 receptor-associated factor may be CD40 receptor-associated factor 1. The antibody may be a monoclonal antibody, a chimeric antibody, or a humanized antibody.

The portion of the antibody may comprise a complementarity determining region or variable region of a light or heavy chain. The portion of the antibody may comprise a complementarity determining region or a variable region, or a Fab.

The present invention provides for a method of differentiating a subject with hyper-IgM syndrome due to an abnormal CD40 receptor-associated factor polypeptide from those with hyper-IgM syndrome due to other causes, comprising: contacting a proteinaceous extract from cells derived from the subject with the antibody or portion thereof under conditions which would permit specific binding of the antibody with normal CD40

- 27 -

receptor-associated factor if present; and detecting the absence of a complex of the antibody with protein in the extract, thereby differentiating a subject with hyper-IgM syndrome due to an abnormal CD40 receptor-associated factor polypeptide from those with hyper-IgM syndrome due to other causes.

The abnormal CD40 receptor-associated factor polypeptide may be abnormal CD40 receptor-associated factor 1. The subject may express CD40 ligand normally. The subject may be a human. The subject may be a murine animal. The cell may be a B cell. The cell may be derived from a cell culture, or from a bodily fluid.

The abnormal CD40 receptor-associated factor polypeptide may be truncated. The abnormal CD40 receptor-associated factor may be truncated at the carboxy terminus, or by at least about 170 amino acid residues, or by at least about 244 amino acid residues, or at the amino terminus, or by at least about 100 amino acid residues.

The antibody or portion thereof may be labeled. The label may be a radioactive isotope such as iodine-125.

The present invention also provides a method for killing a tumor cell which comprises contacting the tumor cell with a CRAF1 peptide or variant or isoform thereof so as to activate CD40 signaling and thereby kill the tumor cell. The tumor cell may be a breast tumor cell, a prostate cancer tumor cell, a liver cancer cell, a lung cancer cell, a nasoepithelial cancer cell, an EBV induced tumor cell, a nasopharyngeal tumor cell, an esophageal cancer cell, a colon cancer cell, a gastric cancer cell, a central nervous system tumor cell, an ovarian cancer cell, or a cervical cancer cell. CD40 signaling may be used as a signal for death in transformed cells (Hess, S. and H. Engelmann. 1996. J.

- 28 -

Exp. Med. 183:159).

This invention provides for a method of performing anti-tumor therapy with CRAF1: CD40 signaling kills tumor cells by apoptosis, particularly when the tumor cell is a non-B cell. This invention provides for a method for treating a subject suffering from a neoplastic condition which comprises administration of a CRAF1 peptide or a variant or an isoform thereof in an effective amount so as to kill a tumor cell and thereby treat the neoplastic condition in the subject. The tumor cell may be stimulated to undergo apoptosis.

The cell may be in a subject suffering from cancer and the CRAF1 peptide or variant or isoform thereof is delivered to the tumor cell.

In an embodiment of the method of inhibiting activation by CD40 ligand of cells bearing CD40 on the cell surface, the cells are provided with the protein of this invention by introducing into the cells a nucleic acid sequence encoding the protein under conditions such that the cells express an amount of the protein effective to inhibit activation of the cells. The nucleic acid may be DNA (including cDNA) or RNA. It may be single or double stranded, linear or circular. It may be in the form of a vector such as a plasmid or a viral vector. Preferably the nucleic acid sequence is operably linked to a transcriptional control sequence recognized by the host cell.

In embodiments of the methods described herein, the CD40-bearing cells are selected from the group consisting of B cells, fibroblasts, endothelial cells, epithelial cells, T cells, basophils, macrophages, Reed-Steinberg cells, dendritic cells, myeloma cells, renal cells, and smooth muscle cells.

- 29 -

In a more specific embodiment the B cells are resting B cells, primed B cells, myeloma cells, lymphocytic leukemia B cells, or B lymphoma cells. In another specific embodiment the epithelial cells are keratinocytes. In another embodiment the fibroblasts are synovial membrane fibroblasts, dermal fibroblasts, pulmonary fibroblasts, or liver fibroblasts. In another specific embodiment the renal cells are selected from the group consisting of glomerular endothelial cells, mesangial cells, distal tubule cells, proximal tubule cells, parietal epithelial cells (e.g., crescent parietal epithelial cells), visceral epithelial cells, cells of a Henle limb, and interstitial inflammatory cells. In another embodiment the smooth muscle cells are smooth muscle cells of the bladder, vascular smooth muscle cells, aortic smooth muscle cells, coronary smooth muscle cells, pulmonary smooth muscle cells, or gastrointestinal smooth muscle cells. In a more specific embodiment the gastrointestinal smooth muscle cells are esophageal smooth muscle cells, stomachic smooth muscle cells, smooth muscle cells of the small intestine, or smooth muscle cells of the large intestine.

This invention provides a method of providing a subject with an amount of the protein of this invention effective to inhibit activation by CD40 ligand of cells bearing CD40 on the cell surface in the subject, comprising: introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding the protein of this invention, under conditions such that the cells express in the subject an activation inhibiting effective amount of the protein.

In an embodiment of this invention the introducing of the nucleic acid into cells of the subject comprises: a) treating cells of the subject ex vivo to insert the

- 30 -

nucleic acid sequence into the cells; and b) introducing the cells from step a) into the subject.

5 The subject which can be treated by the methods described herein is an animal. Preferably the animal is a mammal. Subjects specifically intended for treatment with the method of the invention include humans, as well as nonhuman primates, sheep, horses, cattle, goats, pigs, dogs, cats, rabbits, guinea pigs, hamsters, 10 gerbils, rats and mice, as well as the organs, tumors, and cells derived or originating from these hosts.

This invention provides a method of treating a condition characterized by an aberrant or unwanted level of CD40-mediated intracellular signaling, in a subject, 15 comprising providing the subject with an amount of the peptide of this invention capable of inhibiting CD40-mediated intracellular signaling in cells bearing CD40 on the cell surface. The peptide of the present invention may be a protein. 20

In an embodiment the protein is provided by introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding the protein, under conditions such 25 that the cells express in the subject an activation inhibiting effective amount of the protein.

In an embodiment the condition is organ rejection in a subject receiving transplant organs. Examples of 30 suitable transplant organs include a kidney, heart or liver, as well as others known to those of skill in the art. In another embodiment the condition is an immune response in a subject receiving gene therapy. One difficulty encountered in gene therapy is an immune response by the patient to the gene therapy vector and 35 the proteins it expresses (Yang, Y. et al. (1996) J. Virol. 70:6370). Because the protein of this invention

- 31 -

inhibits the immune response, gene therapy with the protein of this invention does not trigger an immune response. Its immunosuppressant effect also makes it useful as an adjunct to other forms of gene therapy.

5 For example, at the same time that a vector being administered to provide a gene therapy patient with a desired gene product, the patient is also administered a vector which provides the protein of this invention.

10 In another embodiment the condition is an allergic response, including but not limited to hay fever or a penicillin allergy, atopic dermatitis and extrinsic asthma.

15 In an embodiment of this invention the immune response comprises induction of CD23, CD80 upregulation, or rescue from CD95-mediated apoptosis. Because CD40, which is expressed by many tumors, is involved in rescuing cells from apoptosis, inhibitors of CD40-

20 mediated activity are useful as adjunctive agents in chemotherapy.

In an embodiment of this invention the immune response is autoimmune manifestations of an infectious disease.

25 In more specific embodiments the autoimmune manifestations are derived from Reiter's syndrome, spondyloarthritis, Lyme disease, HIV infections, syphilis or tuberculosis.

30 In an embodiment the condition is dependent on CD40 ligand-induced activation of fibroblast cells, for example arthritis, scleroderma, and fibrosis. In more specific embodiments the arthritis is rheumatoid arthritis, non-rheumatoid inflammatory arthritis,

35 arthritis associated with Lyme disease, or osteoarthritis. In another specific embodiment the fibrosis is pulmonary fibrosis, hypersensitivity

- 32 -

pulmonary fibrosis, or a pneumoconiosis. Examples of pulmonary fibrosis include pulmonary fibrosis secondary to adult respiratory distress syndrome, drug-induced pulmonary fibrosis, idiopathic pulmonary fibrosis, or hypersensitivity pneumonitis. Examples of pneumoconiosis include asbestosis, siliconosis, or Farmer's lung. In another specific embodiment the fibrosis is a fibrotic disease of the liver or lung, including fibrotic disease of the lung caused by rheumatoid arthritis or scleroderma, and fibrotic diseases of the liver selected from the group consisting of: Hepatitis-C; Hepatitis-B; cirrhosis; cirrhosis of the liver secondary to a toxic insult; cirrhosis of the liver secondary to drugs; cirrhosis of the liver secondary to a viral infection; and cirrhosis of the liver secondary to an autoimmune disease. In a specific embodiment the toxic insult is alcohol consumption. In another specific embodiment the viral infection is Hepatitis B, Hepatitis C, or hepatitis non-B non-C. In another specific embodiment the autoimmune disease is primary biliary cirrhosis, or Lupoid hepatitis.

In an embodiment of this method the condition is dependent on CD40 ligand-induced activation of endothelial cells. In specific embodiments the condition dependent on CD40 ligand-induced activation of endothelial cells is selected from the group consisting of atherosclerosis, reperfusion injury, allograft rejection, organ rejection, and chronic inflammatory autoimmune diseases. In a more specific embodiment the atherosclerosis is accelerated atherosclerosis associated with organ transplantation. In another specific embodiment the chronic inflammatory autoimmune disease is vasculitis, rheumatoid arthritis, scleroderma, or multiple sclerosis.

In an embodiment the condition is dependent on CD40

- 33 -

ligand-induced activation of epithelial cells. In a specific embodiment the epithelial cells are keratinocytes, and the condition is psoriasis. In another specific embodiment the condition is an inflammatory kidney disease, including inflammatory kidney disease not initiated by autoantibody deposition in kidney and kidney disease which is initiated by autoantibody deposition. In specific embodiments the kidney disease is selected from the group consisting of: membranous glomerulonephritis; minimal change disease/acute tubular necrosis; pauci-immune glomerulonephritis; focal segmental glomerulosclerosis; interstitial nephritis; antitissue antibody-induced glomerular injury; circulating immune-complex disease; a glomerulopathy associated with a multisystem disease; and drug-induced glomerular disease. In an embodiment the antitissue antibody-induced glomerular injury is anti-basement membrane antibody disease. In another embodiment the circulating immune-complex disease is selected from the group consisting of: infective endocarditis; leprosy; syphilis; hepatitis B; malaria; and a disease associated with an endogenous antigen. In a more specific embodiment the endogenous antigen is DNA, thyroglobulin, an autologous immunoglobulin, erythrocyte stroma, a renal tubule antigen, a tumor-specific antigen, or a tumor-associated antigen. In another embodiment the glomerulopathy associated with a multisystem disease is selected from the group consisting of: diabetic nephropathy; systemic lupus erythematosus; Goodpasture's disease; Henoch-Schönlein purpura; polyarteritis; Wegener's granulomatosis; cryoimmunoglobulinemia; multiple myeloma; Waldenström's macroglobulinemia; and amyloidosis. In an embodiment the pauci-immune glomerulonephritis is ANCA+ pauci-immune glomerulonephritis, or Wegener's granulomatosis. In an embodiment the interstitial nephritis is drug-induced interstitial nephritis. In another embodiment

- 34 -

the kidney disease affects renal tubules, including but not limited to: a kidney disease associated with a toxin; a neoplasia; hypersensitivity nephropathy; Sjögren's syndrome; and AIDS.

5

In an embodiment the condition is a smooth muscle cell-dependent disease. Examples include vascular diseases such as atherosclerosis; gastrointestinal diseases such as esophageal dysmotility, inflammatory bowel disease, and scleroderma; and bladder diseases.

10

In an embodiment of this method, the condition is associated with Epstein-Barr virus. Examples of Epstein-Barr virus-associated conditions include mononucleosis, B cell tumors (particularly in immunosuppressed individuals such as chemotherapy patients and those with acquired immune deficiency syndrome (AIDS)), Burkitt's lymphoma, and nasopharyngeal carcinoma. Epstein-Barr virus (EBV) transforms cells using latent infection membrane protein 1 (LMP1). LMP1 binds to CRAF1 (also known as LAP1) (33).

15

20

Inhibition of the immune response using an antibody against CD40L may potentially making the patient susceptible to pneumocystis pneumonia. Hyper IgM patients who have defective B cell activation signaling, do not get pneumocystis pneumonia, other atypical infections, and tumors. The data presented herein indicate that people with the 70 kDa isoform of CRAF1 are a subset of these patients. Accordingly, in an embodiment of the above-described method of treating a subject, the treatment does not increase susceptibility of the subject to atypical infections (e.g., pneumocystis pneumonia) or tumors.

25

30

35

This invention provides a nucleic acid molecule encoding the protein of this invention. The nucleic acid may be

- 35 -

DNA (including cDNA) or RNA. It may be single or double stranded, linear or circular. It may be in the form of a vector, such as a plasmid or viral vector, which comprises the nucleic acid molecule operably linked to a transcriptional control sequence recognized by a host cell transformed with the vector. In one embodiment the DNA molecule comprises the coding strand of the CRAF1 IIIb clone (Figures 1A-1P). In another embodiment the DNA molecule is complementary to the coding strand of the CRAF1 IIIb clone. In a specific embodiment the plasmid is pBluescriptSKII+/IIIb.

This invention provides an isolated CRAF1 protein or variant thereof comprising from zero to four zinc fingers; wherein when the protein comprises zinc fingers 1, 2 and 5, the protein further comprises one or both of zinc fingers 3 and 4.

The numbering of CRAF1 zinc finger domains is based on the schematic shown in Figure 4. The zinc finger domains are numbered consecutively from 1 to 5, proceeding from the amino terminus to the carboxy terminus. The zinc fingers, if any, in the protein of this invention, may be present in the order in which they appear in wild-type CRAF-1 (e.g., N-1, 3, 4, 5-C) or in a different order (e.g. N-1, 4, 5-C). A zinc finger domain can be eliminated by deleting the corresponding exon or part of the exon, or by other mutagenesis techniques, for example a point mutation which changes a zinc finger cysteine or histidine to another amino acid which disrupts the zinc finger domain structure.

CRAF1 proteins or variants thereof comprising from zero to four zinc finger domains of this invention may be capable of inhibiting CD40-mediated cell activation in vitro and in vivo.

- 36 -

In an embodiment of this invention the protein comprises zero, one, two or four zinc fingers. In another embodiment, the protein comprises CRAF1 or variant thereof deleted by zinc finger domains 2, 3 and 4, or by
5 exons 8, 9, and 10 in the zinc finger domain encoding region. In another embodiment the protein comprises CRAF1 or variant thereof deleted by amino acids 191 to 242. In specific embodiments the protein is CRAF1a deleted by amino acids 191 to 242 or CRAF1b deleted by
10 amino acids 313 to 364. The deletions of these amino acids result in the formation of new zinc finger domains comprised of different groups of amino acids.

In an embodiment of this invention, the variant
15 comprises a conservative amino acid substitution. Variants can differ from naturally occurring CRAF1 and isoforms thereof comprising from one to four zinc fingers, in amino acid sequence or in ways that do not involve sequence, or both.

20 This invention provides a method of inhibiting activation by CD40 ligand of cells bearing CD40 on the cell surface, comprising providing the cells with an isolated CRAF1 protein or variant thereof comprising
25 from zero to four zinc fingers, the protein being present in an amount effective to inhibit activation of the cells.

In an embodiment of the method of inhibiting activation
30 by CD40 ligand of cells bearing CD40 on the cell surface, the cells are provided with isolated CRAF1 protein or variant thereof comprising from zero to four zinc fingers by introducing into the cells a nucleic acid sequence encoding the protein under conditions such
35 that the cells express an amount of the protein effective to inhibit activation of the cells. The nucleic acid may be DNA (including cDNA) or RNA. It may

- 37 -

be single or double stranded, linear or circular. It may be in the form of a vector such as a plasmid or a viral vector. Preferably the nucleic acid sequence is operably linked to a transcriptional control sequence recognized by the host cell.

This invention provides a method of providing a subject with an amount of isolated CRAF1 protein or variant thereof comprising from zero to four zinc fingers effective to inhibit activation by CD40 ligand of cells bearing CD40 on the cell surface in the subject, comprising: introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding the protein, under conditions such that the cells express in the subject an activation inhibiting effective amount of the protein.

In one embodiment, the protein comprises CRAF1 or variant thereof, deleted by amino acids 218 to 242. In specific embodiments the protein is CRAF1a deleted by amino acids 218 to 242 or CRAF1b deleted by amino acids 340 to 364. In another embodiment the protein comprises CRAF1 or variant thereof, deleted by amino acids 191 to 242. In specific embodiments the protein is CRAF1a deleted by amino acids 191 to 242 or CRAF1b deleted by amino acids 313 to 364.

In an embodiment of this invention the introducing of the nucleic acid into cells of the subject comprises: a) treating cells of the subject ex vivo to insert the nucleic acid sequence into the cells; and b) introducing the cells from step a) into the subject.

This invention provides a method of treating a condition characterized by an aberrant or unwanted level of CD40-mediated intracellular signaling, in a subject, comprising providing the subject with an amount of an

- 38 -

isolated CRAF1 protein or variant thereof comprising from zero to four zinc fingers capable of inhibiting CD40-mediated intracellular signaling in cells bearing CD40 on the cell surface.

5

In an embodiment the protein is provided by introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding the protein, under conditions such that the cells express in the subject an activation
10 inhibiting effective amount of the protein.

In another embodiment the condition is a CD40-dependent immune response. In a specific embodiment the CD40-dependent immune response is an autoimmune response in
15 a subject suffering from an autoimmune disease, including but not limited to rheumatoid arthritis, Myasthenia gravis, systemic lupus erythematosus, Graves' disease, idiopathic thrombocytopenia purpura, hemolytic anemia, diabetes mellitus, a drug-induced autoimmune
20 disease such as drug-induced lupus, psoriasis, hyper IgE syndrome, or abdominal aortic aneurism (AAA).

This invention provides a nucleic acid molecule encoding the peptide of this invention. The nucleic acid may be
25 DNA (including cDNA) or RNA or mRNA. It may be single or double stranded, linear or circular. It may be in the form of a vector, such as a plasmid or viral vector, which comprises the nucleic acid molecule operably linked to a transcriptional control sequence recognized
30 by a host cell transformed with the vector. In one embodiment the nucleic acid molecule encodes a CRAF1 peptide that does not contain exon 9 (**Figures 1A-1P**). The nucleic acid can be a coding strand or complementary to the coding strand or a reverse complement to the
35 coding strand. In specific embodiments the CRAF1 nucleic acid molecule may be any of the specific sequences shown in Figures 1A-1P and in Tables 1 and 2.

- 39 -

This invention provides a method of providing a subject with an amount of a CRAF1 protein or variant thereof comprising from zero to four zinc fingers, effective to inhibit activation by CD40 ligand of cells bearing CD40 on the cell surface in the subject, comprising:
5 introducing into CD40-bearing cells of the subject an agent capable of enhancing RNA splicing, under conditions such that the cells express in the subject an activation inhibiting effective amount of the protein.

10

In one embodiment the agent is a splicing apparatus element.

15

In embodiments of this method, the element is a protein; an RNA, for example a small nuclear RNA (snRNA); or a small nuclear ribonucleoprotein (snRNP). In another embodiment the agent is a vector encoding a splicing apparatus element.

20

The agent can enhance nonspecific RNA splicing, or it can specifically affect CRAF1 RNA splicing. In embodiments of this invention the agent enhances downstream splicing, or upstream splicing.

25

In an embodiment of this invention the agent enhances formation of a spliceosome, wherein the spliceosome comprises pre-mRNA encoding the CRAF1 protein or variant thereof comprising from zero to four zinc fingers.

30

This invention provides a method of differentiating a subject with hyper-IgM syndrome due to an abnormal CD40 receptor-associated factor polypeptide from those with hyper-IgM syndrome due to other causes, comprising:
35 detecting in an extract from a cell derived from the subject, the presence of a nucleic acid encoding the abnormal CD40 receptor-associated factor, thereby differentiating the subject with hyper-IgM syndrome due

- 40 -

to an abnormal CD40 receptor-associated factor polypeptide from those with hyper-IgM syndrome due to other causes.

- 5 In a preferred embodiment the abnormal CD40 receptor-associated factor polypeptide is abnormal CD40 receptor-associated factor 1.

10 As described in the Experimental Methods section below, the expression of mutant or truncated forms of CRAF! Can serve as a dominant negative. Accordingly, in a specific embodiment the subject is heterozygous for the abnormal CD40 receptor-associated factor.

- 15 In an embodiment of this invention the subject with hyper-IgM syndrome expresses CD40 ligand normally.

20 In a preferred embodiment the cell from which the extract is derived is a B cell. The cell may be derived from a cell culture or from a bodily fluid, including but not limited to blood.

25 The gene encoding abnormal CRAF may contain point mutations, frame shift mutations, a premature stop codon. Alternatively, it may be missing a segment of the gene at the 5' or 3' end of the coding region. In an embodiment of this invention the nucleic acid encodes a truncated CD40 receptor-associated factor polypeptide. In a specific embodiment the the nucleic acid encodes the CD40 receptor-associated factor truncated at the carboxy terminus. In more specific embodiments the carboxy-terminal truncated CRAF is truncated by at least about 170 amino acid residues, or by at least about 244 amino acid residues. In another specific embodiment the
30 nucleic acid encodes the CD40 receptor-associated factor truncated at the amino terminus. In a more specific embodiment the amino-terminal truncated CRAF is
35

- 41 -

truncated by at least about 171 amino acid residues or 258 amino acids.

5 The nucleic acid present in the extract can be RNA or DNA. It can be single stranded or double stranded. In an embodiment the nucleic acid in the extract is mRNA. In another embodiment the nucleic acid in the extract is DNA, including single stranded and double stranded DNA.

10 In an embodiment the nucleic acid is amplified prior to detecting, and the detecting is detecting of the amplified nucleic acid. When the nucleic acid is mRNA a cDNA copy is preferably made by reverse transcription. Reverse transcriptase is commercially available, for
15 example from New England Biolabs (Beverly, MA). PCR, a well known laboratory technique (See for example Sambrook, et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, 1989) ch. 14), is the preferred for amplifying the nucleic acid.

20 In one embodiment of this invention the detecting comprises: contacting the nucleic acid to be detected with a probe, wherein, if the nucleic acid to be detected is DNA, the probe is capable of hybridizing to
25 a coding or noncoding strand of a unique sequence encoding a normal CD40 receptor-associate factor; and if the nucleic acid to be detected is RNA, the probe is capable of hybridizing to a unique sequence encoding a normal CD40 receptor-associated factor, under stringent
30 conditions which would permit hybridization with the unique sequence if present; and detecting the absence of a hybrid of the probe and the nucleic acid to be detected, thereby detecting the nucleic acid encoding the abnormal CD40 receptor-associated factor. The
35 length of the probe should be chosen to provide a high probability that it will recognize a unique sequence. This will depend in part on the length of the genome of

- 42 -

the subject and can be easily calculated by one of skill in the art to which this invention pertains. In specific embodiments the probe comprises at least nine nucleotides, at least twelve nucleotides, or at least fifteen nucleotides. In an embodiment the probe is labeled. In a specific embodiment the label is a radioactive isotope. A number of suitable radioactive isotopes are known to those of skill in the art, including but not limited to iodine-125.

This invention also provides a method of providing a subject with an immunosuppressant effective amount of an abnormal CD40 receptor-associated factor, comprising: introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding an abnormal CD40 receptor-associated factor polypeptide, under conditions such that the cells express in the subject an immunosuppressant effective amount of the abnormal CD40 receptor-associated factor.

Gene therapy for providing a subject with a protein encoded by a gene are described in U.S. Patent No. 5,399,346, issued March 21, 1995 (Anderson, et al.). A nucleic acid sequence encoding the protein of interest can be inserted into cells of the subject in vivo. Alternatively the nucleic acid can be inserted into cells ex vivo and the transfected cells can then be introduced into the subject. Accordingly, in an embodiment the introducing of the nucleic acid into cells of the subject comprises: a) treating cells of the subject ex vivo to insert the nucleic acid sequence into the cells; and b) introducing the cells from step a) into the subject.

In an embodiment of this invention the abnormal CD40 receptor-associated factor polypeptide is abnormal CD40 receptor-associated factor 1.

- 43 -

In an embodiment of this invention the nucleic acid sequence encodes a truncated CD40 receptor-associated factor polypeptide. In a specific embodiment the nucleic acid sequence encodes CD40 receptor-associated factor polypeptide truncated at the amino terminus. In a more specific embodiment the amino-terminal truncated CD40 receptor-associated factor polypeptide is truncated by at least about 171 amino acid residues. In another specific embodiment the amino-terminal truncated CD40 receptor-associated factor polypeptide is truncated by at least about 323 amino acid residues. In another specific embodiment the nucleic acid sequence encodes CD40 receptor-associated factor polypeptide truncated at the carboxy terminus. In a more specific embodiment the carboxy-terminal truncated CD40 receptor-associated factor polypeptide is truncated by at least about 258 amino acid residues.

This invention provides a method of inhibiting a CD40-dependent immune response in a subject, comprising providing the subject with an immunosuppressant effective amount of an abnormal CD40 receptor-associated factor by a method which comprises introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding an abnormal CD40 receptor-associated factor polypeptide, under conditions such that the cells express in the subject an immunosuppressant effective amount of the abnormal CD40 receptor-associated factor as described above.

In specific embodiments the immune response which is inhibited comprises induction of CD23, CD80 upregulation, or rescue from CD95-mediated apoptosis.

This invention also provides an antibody or portion thereof capable of specifically binding to a CD40 receptor-associated factor.

Techniques for producing antibodies are well known in the art and are described in standard laboratory manuals, such as Harlow and Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor 1988) pp. 53-138. The CRAF protein, whether purified or recombinant, is preferably coupled to a carrier protein. Examples of useful carrier proteins include keyhole limpet hemacyanin, ovalbumin, bovine serum albumin, mouse serum albumin, and rabbit serum albumin. The CRAF, alone or coupled to a carrier protein, is injected into an animal, for example a mouse or rabbit in order to immunize it. Antibodies against the CRAF are then isolated from the immunized animal

In a specific embodiment of this invention the CD40 receptor-associated factor is CD40 receptor-associated factor 1.

The antibody may be either a polyclonal or monoclonal antibody. The production of monoclonal antibodies using hybridoma technology are well known to those of skill in the art and are described in standard laboratory manuals, such as Harlow and Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor 1988) pp. 139-282.

In specific embodiments the monoclonal antibody is a chimeric antibody or a humanized antibody. The meaning of "chimeric" and "humanized" antibody and methods of producing them are well known to those of skill in the art and are described, for example, in PCT International Publication No. WO 90/07861, published July 26, 1990 (Queen, et al.); and Queen, et al. Proc. Nat'l Acad. Sci.-USA (1989) 86: 10029.

In another embodiment the portion of the antibody comprises a complementarity determining region or

- 45 -

variable region of a light or heavy chain. In yet another embodiment the portion of the antibody comprises a complementarity determining region or a variable region. In an embodiment the portion of the antibody
5 comprises a Fab.

This invention provides a method of differentiating a subject with hyper-IgM syndrome due to an abnormal CD40 receptor-associated factor polypeptide from those with
10 hyper-IgM syndrome due to other causes, comprising: contacting a proteinaceous extract from cells derived from the subject with the antibody or portion thereof as described above under conditions which would permit specific binding of the antibody with normal CD40
15 receptor-associated factor if present; and detecting the absence of a complex of the antibody with protein in the extract, thereby differentiating a subject with hyper-IgM syndrome due to an abnormal CD40 receptor-associated factor polypeptide from those with hyper-IgM syndrome
20 due to other causes.

In an embodiment the abnormal CD40 receptor-associated factor polypeptide is abnormal CD40 receptor-associated factor 1.
25

In an embodiment the subject expresses CD40 ligand normally.

In an embodiment of this invention the abnormal CD40 receptor-associated factor polypeptide is truncated at
30 the carboxy terminus or the amino-terminus. In a specific embodiment the carboxy-terminal truncated abnormal CD40 receptor-associated factor polypeptide is truncated by at least about 171 amino acid residues. In
35 another specific embodiment the abnormal CD40 receptor-associated factor polypeptide is truncated by at least about 244 amino acid residues. In another specific

- 46 -

embodiment the amino-terminal truncated abnormal CD40 receptor-associated factor polypeptide is truncated by at least about 258 amino acid residues.

5 In an embodiment of this invention the antibody or portion thereof is labeled. In a specific embodiment the antibody or portion thereof is labeled with a radioactive isotope. Suitable radioisotopes are known to those of skill in the art and include, but are not
10 limited to iodine-125.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific
15 methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

- 47 -

Experimental Details

The following two tables (Tables 1 and 2) provide some details about several embodiments of the present invention such as exon structure, splice donor and splice acceptor sites, base pair number, etc.

Table 1. Structure of TRAF-3 mRNAs and Putative Peptide Isoforms

Exons

10	Isoforms	1	2	3	4	5	6	7	8	9	10	11 -13
	TRAF-3 (p70)	x	x		x	x	x	x	x	x	x	x
	TRAF-3 (p70) del-9	x	x		x	x	x	x	x		x	x
	TRAF-3 (p70) del-8,9	x	x		x	x	x	x			x	x
	TRAF-3 (p55) del-9,10	x	x		x	x	x	x	x			x
15	2-1 Type Transcripts *											
	TRAF-3 (p55) *	x		x	x	x	x	x	x	x	x	x
	TRAF-3 (p55) del-9	x		x	x	x	x	x	x		x	x
	TRAF-3 (p55) del-8,9	x		x	x	x	x	x			x	x
20	TRAF-3 (p55) del-9,10	x		x	x	x	x	x	x			x
	Ib type transcripts **											
	TRAF-3 (p55) **	x	x	x	x	x	x	x	x	x	x	x
	TRAF-3 (p55) del-9***	x	x	x	x	x	x	x	x		x	x
25	TRAF-3 (p55) del-8,9	x	x	x	x	x	x	x			x	x
	TRAF-3 (p55) del-9.10	x	x	x	x	x	x	x	x			x

*Previously reported as CRAF-1, LAP-1

**Previously reported as CD40-bp

***Previously reported as CAP-1

+ 2-1 type transcripts also encode TRAF-3 (p5)

++Ib type transcripts also encode TRAF-3 (p15)

Note: exon 8 encodes aa 191-217 of TRAF-3 (p55)

exon 9 encodes aa 218-242 of TRAF-3 (p55)

exon 10 encodes aa 242-273 of TRAF-3 (p55)

35

Table 2. Genomic Structure of TRAF-3

up stream exon	nt	lib num- bering	size (bp)	splice acceptor	splice donor	down stream size (kb)	intron class*
1	1-309	---	309		CCACCCgtgagcaagaca (Seq I.D. No. ____)	0.793	0
2	310-517	---	208	tgcgtgagggagCGAGGG (Seq I.D. No. ____)	CGCAAGgttaangggccg (Seq I.D. No. ____)	54.5	1
3	518-657	---	139	tttatttttacagATGAGG (Seq I.D. No. ____)	GAAGAGgtttgctctcag (Seq I.D. No. ____)	>28	-
4	658-918	207-468	262	ttttcccgacagAACTCC (Seq I.D. No. ____)	GCTGAGgttaggcgccttc (Seq I.D. No. ____)	1.7	2
5	919-971	469-520	52	tttgccctgcagCTCTTC (Seq I.D. No. ____)	GATAAGgtattctggggt (Seq I.D. No. ____)	3.5	0
6	972-1076	521-625	105	tttcattttcagGTGTTT (Seq I.D. No. ____)	CTGCTGgtGagtagcaaa (Seq I.D. No. ____)	0.600	0
7	1077-1244	626-793	168	tctgtcttacagGTGCAT (Seq I.D. No. ____)	CTGCAGgtgcggtccttc (Seq I.D. No. ____)	9.5	0
8	1245-1325	794-874	81	ctctctctgtagAAACAC (Seq I.D. No. ____)	AGCGAGgtagggcgggcc (Seq I.D. No. ____)	4.0	0
9	1326-1400	875-949	75	ttcccggtgcagTTGAGT (Seq I.D. No. ____)	TTTCAGgtcagtatccga (Seq I.D. No. ____)	1.8	0
10	1401-1493	950-1042	93	ttgctctgcagGGGACA (Seq I.D. No. ____)	AAGAAGgtgggctgcaca (Seq I.D. No. ____)	6	0
11	1494-1634	1043-1183	142	tttcttttttagGTTTCC (Seq I.D. No. ____)	TTACAGgtaagaatctta (Seq I.D. No. ____)	4.5	0
12	1635-1809	1184-1358	174	ttggtttggaagCGAGTG (Seq I.D. No. ____)	ACACAGgtgaggcagggg (Seq I.D. No. ____)	2	1
13	1810-2381	1359-1930	572	cacctgtggcagGCCTGC (Seq I.D. No. ____)	poly-A tail (stop at 1928)		

Note: where ambiguous, boundary assignments optimize gt/ag consensus.

* intron class: 0: jxn falls between codons, 1: jxn falls after 1st nt of codon, 2: jxn falls after 2nd nt of codon.

359-540 Zn ring, 550-1015 Zn fingers, 1080-1250 Helical Wheel, 1290-stop TRAF domain (1470-stop TRAF-C domain)

Example 1: Complementary DNA Encoding Long CRAF1 Isoform

Several cDNA species encoding human CRAF1 nucleic acid from a Raji B cell library (several of which are described in Tables 1 and 2) have been isolated that reveal unexpected complexity in the 5' region of these cDNAs. Although divergent in some cases, comparison of the sequences of these clones suggests at least 8 unique

- 49 -

segments, several of which are shared between the cDNA clones and are therefore highly suggestive of alternative splicing events. Given the identity of several distinct cDNA sequence elements between these clones, alternative splicing may account for at least some of these data. However, the formal possibility exists that CRAF1 peptide is encoded by several distinct genes. From the sequences of these cDNAs, it can be inferred that at least putative eight exons exist. These exons have been assigned number codes (i.e., 1-13). The organization and arrangement of these exons is shown in **Figures 2A and 2B and Figure 3**. **Figures 1A-1P** show the sequences of exons 1-13. The exon boundaries shown in **Figures 1A-1P** are derived from the comparison of cDNA sequences. They may be adjusted by a few nucleotides based on genomic sequencing.

Many of the cDNA species are expected to yield proteins with the published 568 aa sequence of CRAF1 (p55) (75) since an upstream in frame stop codon is present. However, at least one of these species, represented by cDNA clone IIIb (ATCC Accession No. 97489) contains an open reading frame that is not interrupted by a stop codon (**Figures 1A-1P**) and Genbank Accession number U21092). The predicted amino acid sequence of the IIIb clone preceding the published

MESSKKD - start site is: (-71-
GARRGRVREPGLQPSRDFPAGGSRGRRLLFPAPRHGAARGA(E/K)(R/C)CG
PRR(Q/R)TRPAPLSRPSGDGP(Q/R)ELLFPK-1)(Seq I.D. No. ____).

The structure of this domain is highly charged as indicated by the presence of 18/71 arginine residues. It also appears to be an extended strand as evidenced by the presence of 13/71 glycines and 11/71 prolines. In fact, the arginine, glycine and proline content of this putative sub-domain comprises 42/71 of the residues. It does not have homology to any other sequence in GenBank, and does not have an identifiable motif that suggests

- 50 -

its function. Two arginine and proline-rich domain motifs have been described (SH₃-binding domain, and WW domain). This sequence does not appear to be either of these two, based on amino acids -71 to -1.

5

However, the new amino-terminal domain of TRAF-3 p70 described by the first 71 aa has some features of an Src homology 3 (SH3)-binding domain protein (Alexandropoulos, K., et al. (1995) PNAS, 92:3110; Cohen, G. B., et al. 1995. Cell 80:237). The sequence aa 103-RPAPLSRP-110 (present in both p70-i and p15) is similar to the RPLPXXP motif that accounts for SH3-binding in other proteins. In addition, by the criteria of the PXXP motif, (Saksela, K., et al. 1995. EMBO Journal 14:484), the novel amino terminal domain of p70 (that is shared by p5 and p15) contains the sequences: 16-PQRP-19 and 44-PCPP-47.

As used herein, patient H is the same as patient A; patient L is the same as patient B; patient S is the same as patient D; mother of patient C is the same as CM; mother of patient D is the same as DM and the same as RS.

To determine if the IIIB-type cDNA (CRAF1 peptide, TRAF-3-p55) is represented by mRNA in normal and hyper IgM B cells that have been EBV-transformed, RT-PCR was performed on mRNA from three EBV transformed B cell lines; one from a normal subject and two from the HIM patients A and B, by priming the RT reaction with 3'-reverse primer and amplifying with the primer pair oCRf(127-144)/oCRr(675-658) (predicted product (127-675) for a 548 bp product). The oCRf(127-144) anneals to a region in exon 2 and oCRr(675-658) anneals to the conserved exon 4-13 region shared by all cDNAs. (These nucleotide numbers refer to nucleotides in the sequence shown in **Figures 1A-1P**. Primers in which the first

- 51 -

number is higher than the second are reverse nucleotide primers.) Two bands were excised from the gel, cloned in the TA system (pCRII, Invitrogen®) and sequenced. The amplified 548 bp band corresponds exactly to sequence of the [exons 2,4-13] product (analogous to IIIb). These results strongly suggest that species of the [exons 2, 4-13] product are found in B cells. In addition, these data suggest that the protein isoform of which the [exons 2, 4-13] transcript is a partial cDNA clone of a species that exists in normal cells. Thus, this protein isoform is termed the CRAF1-b isoform (TRAF-3-p-70), in contrast to the protein isoform encoded by transcript which initiates translation at methionine 1 (nucleotides 675-678 of Figures 1A-1P), which is termed CRAF1-a (TRAF-3-p-55).

Characterization of CRAF1 Peptides

Immunoprecipitation of metabolically labelled Ramos cells, Ramos/pCEP/CRAF1 and EBV-transformed B cell lines was performed using rabbit antisera against the peptide representing CRAF1 peptide amino-terminus. Several bands are specifically immunoprecipitated by the anti-CRAF1 antisera, including major bands that migrate at 70 kDa and 60 kDa. The intensity of the 60 kDa band is strongly increased in lysates from Ramos/pCEP/CRAF1 cells, which suggests that this band represents the peptide product of the CRAF1-a transcript and which is consistent with the predicted molecular weight of a 568 aa peptide. The expression of both the 60 kDa band and the 70 kDa band is greatly increased in the EBV transformed cells relative to the intensity of these bands in Ramos controls suggesting that EBV transformation increases the expression of CRAF1 peptides and which is consistent with the observation by Kieff and coworkers that EBV upregulates CRAF1 mRNA in BJAB cells (98).

- 52 -

CRAF1-encoded peptides in two unrelated HIM patients

Defective CD40-L molecules underlie a genetic immune deficiency syndrome, the X-linked hyper-IgM syndrome (HIGMX-1), in which B cell differentiation is blocked at the IgM expressing stage and in which germinal centers are absent (12-21). Furthermore, the essential roles of CD40-L in the IgG⁺IgA⁺IgE⁺ phenotype of HIM syndrome has been confirmed by targeted disruption of CD40-L in mice (23,24). However, HIM syndrome does not always involve defects in CD40-L (74,22). In mice, targeted disruption of CD40 results in a phenotype similar to HIM (25,26).

Patients A and B have normal CD40-L expression by the mAb 5c8 and normal expression of CD40 by the mAb G28-5. Both patients A and B are relatively healthy, since they are 49 and 27 years old, respectively, and have not developed neoplastic disease, nor has either had pneumocystis, opportunistic infections or neutropenia, which are features of HIGMX-1 (74).

In order to test whether these hyper IgM patients have a defect in their CRAF1 nucleic acid or their CRAF1 peptide, the peptide products of the CRAF1 gene were analyzed using a rabbit antiserum generated to the NH₂-terminus of the CRAF1 peptide. Immunoprecipitation followed by Western blotting with the CRAF1-specific antisera from EBV-transformed B cell lines from a normal individual and from the hyper-IgM patients A, B and C was performed (**Figure 5**). The anti-CRAF1 antisera specifically identified peptide species that migrate at 70 kDa and 60 kDa. The expression of the p60 CRAF1 is comparable in all the cell lines examined except the EBV-transformed B cells from patient B, in which only trace amounts of the p60 CRAF1 was detected. This result shows that p60 CRAF1 is normally expressed in B lymphocytes. A high molecular weight CRAF1 peptide species, p70 CRAF1 (TRAF-3-p70), was identified in EBV-

- 53 -

transformed B cells (lanes 9-12), but not in non-EBV-transformed cell lines. However the expression level of p70 CRAF1 varies. In EBV-transformed B cells established from normal B lymphocytes, p70 CRAF is only expressed at low levels (lane 9). However, in EBV-transformed B cells from patient A, the expression of p60 CRAF and p70 CRAF1 is comparable (lane 10). Moreover, in EBV-transformed B cells from patient B, the expression of p70 CRAF1 is not only dominant but also dramatically up-regulated. Furthermore, in patient B only trace amounts of the p60 CRAF1 was detected (lane 11). In contrast to hyper IgM patients A and B (which are known to have normal CD40L) in EBV-transformed cells established from patient C, the p70 CRAF1 expression is below the level of detection (lane 12).

The lysates from all the cells examined (except for those from patient C) contain both protein species, suggesting that if these patients have a mutated CRAF1 nucleic acid allele, a normal CRAF1 nucleic acid allele may also be present. The presence of increased amounts of p70 in these two hyper IgM patients (patients A and B) suggests that the p70 protein is an inhibitory protein relative to CD40 signaling.

25

Sequencing Larger CRAF1 Isoforms and cDNA

This invention provides various means for determining the amino acid sequence of CRAF1 peptide isoforms which are longer than CRAF1-b (CRAF1 p70 or TRAF-3-p70), i.e. which have additional amino acid residues N-terminal to residue -72, as well as nucleic acid sequences coding for such longer isoforms. The IIIb clone (p55 CRAF1 nucleic acid) is a partial cDNA clone and that the upstream sequence of full length IIIb-type transcripts do encode a larger peptide. This was confirmed by the identification of an upstream methionine with an appropriate adjacent Kozack sequence.

- 54 -

In one technique, the 70 kDa CRAF1 peptide isoform (CRAF1(p70) or TRAF-3-p70) is isolated using an anti-CRAF1 antibody, and its amino terminus sequence determined. Based on the amino-terminal protein sequence, degenerate oligonucleotides are designed which anneal to the corresponding mRNA, cDNA or genomic DNA. Using standard techniques, these degenerate oligonucleotides are used to clone cDNA that encodes the 70 kDa CRAF1 peptide isoform.

In another technique, using the cDNA sequence of CRAF1 nucleic acid IIIb, cDNA libraries or genomic libraries are screened for DNA sequences upstream of that shown. The encoded amino acid sequence is deduced based on the DNA sequence of the upstream clone.

In another technique, the upstream sequence can be determined using RACE (Rapid Amplification of cDNA Ends). RACE kits are commercially available from BOEHRINGER MANNHEIM® and others. First strand cDNA is synthesized from total or poly(A)+ RNA using a gene specific primer, reverse transcriptase, and a mixture of deoxynucleotides. After purification from unincorporated nucleotides and primers, terminal transferase is used to add a homolypolymeric A-tail to the 3' end of the cDNA. Tailed cDNA is then amplified by PCR using a gene specific primer and an oligo dT-primer. The cDNA obtained thereby is further amplified. It is then sequenced, and the corresponding protein sequence deduced.

In another technique, the upstream sequence is determined using anchored PCR. For example, anchored PCR is used on the template of Raji cDNA library DNA to identify the sequence further 5'-to what is included in the IIIb clone. As a primer in exon 2 in CRAF1 the reverse primer, oCRr(144-126) is used. As anchoring

- 55 -

primers in λ gt11 in separate reactions, any (since the library is not cloned directionally) of the following are used: o λ gf(1033-1056) (5'-ggT ggC gAC gAC TCC Tgg AgC Cgg) (Seq I.D. No. ____), o λ gr(1119-1097) (5'-TTg ACA CCA gAC CAA CTg gTA ATg) (Seq I.D. No. ____), o λ gr(1096-1082) (5'-ggT AgC gAC Cgg CgC) (Seq I.D. No. ____) or o λ gf(998-1015) (5'-CAT ggC TgA ATA TCg Acg) (Seq I.D. No. ____). PCR products are generated using this technique. To determine their relationship to the IIIb-like transcripts, these PCR amplified cDNA fragments are cloned in the TA cloning system (Invitrogen) and sequenced. The sequences are compared to IIIb.

Gene Therapy

The invention features expression vectors for in vivo transfection and expression in particular cell types of CD40 receptor-associated factor truncated at the amino terminus so as to antagonize the function of wild type CD40 receptor-associated factor in an environment in which the wild-type protein is expressed (i.e., introduce abnormal CD40 receptor-associated factor that acts as a dominant negative protein to inhibit CD40 signaling).

Expression constructs of CD40 receptor-associated factor polypeptides may be administered in any biologically effective carrier that is capable of effectively delivering a polynucleotide sequence encoding the CD40 receptor-associated factor to cells in vivo. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, baculovirus, adenovirus, adeno-associated virus and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly, plasmid DNA can be delivered with the help of, for example, cationic liposomes or derivatized (e.g., antibody conjugated) polylysine conjugates, gramicidin

- 56 -

S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO_4 precipitation carried out in vivo.

5

Any of the methods known in the art for the insertion of polynucleotide sequences into a vector may be used. See, for example, Sambrook et al., **Molecular Cloning: A Laboratory Manual**, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and Ausubel et al., **Current Protocols in Molecular Biology**, J. Wiley & Sons, NY (1992), both of which are incorporated herein by reference. Conventional vectors consist of appropriate transcriptional/translational control signals operatively linked to the polynucleotide sequence for a particular anti-fibrotic polynucleotide sequence. Promoters/enhancers may also be used to control expression of anti-fibrotic polypeptide. Promoter activation may be tissue specific or inducible by a metabolic product or administered substance. Such promoters/enhancers include, but are not limited to, the native E2F promoter, the cytomegalovirus immediate-early promoter/enhancer (Karasuyama et al., *J. Exp. Med.*, 169: 13 (1989)); the human beta-actin promoter (Gunning et al., *Proc. Natl. Acad. Sci. USA*, 84: 4831 (1987)); the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al., *Mol. Cell. Biol.*, 4: 1354 (1984)); the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al., **RNA Tumor Viruses**, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1985)); the SV40 early region promoter (Bernoist and Chambon, *Nature*, 290:304 (1981)); the promoter of the Rous sarcoma virus (RSV) (Yamamoto et al., *Cell*, 22:787 (1980)); the herpes simplex virus (HSV) thymidine kinase promoter (Wagner et al., *Proc. Natl. Acad. Sci. USA*, 78: 1441 (1981)); the adenovirus

- 57 -

promoter (Yamada et al., *Proc. Natl. Acad. Sci. USA*, 82: 3567 (1985)).

5 Expression vectors compatible with mammalian host cells for use in gene therapy of tumor cells include, for example, plasmids; avian, murine and human retroviral vectors; adenovirus vectors; herpes viral vectors; and non-replicative pox viruses. In particular, replication-defective recombinant viruses can be
10 generated in packaging cell lines that produce only replication-defective viruses. See **Current Protocols in Molecular Biology**: Sections 9.10-9.14 (Ausubel et al., eds.), Greene Publishing Associates, 1989.

15 Specific viral vectors for use in gene transfer systems are now well established. See for example: Madzak et al., *J. Gen. Virol.*, 73: 1533-36 (1992: papovavirus SV40); Berkner et al., *Curr. Top. Microbiol. Immunol.*, 158: 39-61 (1992: adenovirus); Moss et al., *Curr. Top. Microbiol. Immunol.*, 158: 25-38 (1992: vaccinia virus);
20 Muzyczka, *Curr. Top. Microbiol. Immunol.*, 158: 97-123 (1992: adeno-associated virus); Margulskes, *Curr. Top. Microbiol. Immunol.*, 158: 67-93 (1992: herpes simplex virus (HSV) and Epstein-Barr virus (HBV)); Miller, *Curr. Top. Microbiol. Immunol.*, 158: 1-24 (1992:retrovirus);
25 Brandyopadhyay et al., *Mol. Cell. Biol.*, 4: 749-754 (1984: retrovirus); Miller et al., *Nature*, 357: 455-450 (1992: retrovirus); Anderson, *Science*, 256: 808-813 (1992:retrovirus), all of which are incorporated herein
30 by reference.

Preferred vectors are DNA viruses that include adenoviruses (preferably Ad-2 or Ad-5 based vectors), herpes viruses (preferably herpes simplex virus based
35 vectors), and parvoviruses (preferably "defective" or non-autonomous parvovirus based vectors, more preferably adeno-associated virus based vectors, most preferably

- 58 -

AAV-2 based vectors). See, e.g., Ali et al., *Gene Therapy* 1: 367-384, 1994; U.S. Patent 4,797,368 and 5,399,346 and discussion below.

5 Furthermore, abnormal or wild-type CD40 receptor-associated factor may also be introduced into a target cell using a variety of well-known methods that use non-viral based strategies that include electroporation, membrane fusion with liposomes, high velocity
10 bombardment with DNA-coated microprojectiles, incubation with calcium-phosphate-DNA precipitate, DEAE-dextran mediated transfection, and direct micro-injection into single cells. For instance, an anti-fibrotic polynucleotide encoding an immunosuppressant effective
15 amount of an abnormal CD40 receptor-associated factor may be introduced into a cell by calcium phosphate coprecipitation (Pillicer et al., *Science*, 209: 1414-1422 (1980); mechanical microinjection and/or particle acceleration (Anderson et al., *Proc. Natl. Acad. Sci. USA*, 77: 5399-5403 (1980); liposome based DNA transfer (e.g., LIPOFECTIN-mediated transfection- Fefgner et al., *Proc. Natl. Acad. Sci. USA*, 84: 471-477 (1987), Gao and Huang, *Biochem. Biophys. Res. Comm.*, 179: 280-285, 1991); DEAE Dextran-mediated transfection;
20 electroporation (U.S. Patent 4,956,288); or polylysine-based methods in which DNA is conjugated to deliver DNA preferentially to liver hepatocytes (Wolff et al., *Science*, 247: 465-468 (1990), Curiel et al., *Human Gene Therapy* 3: 147-154 (1992). Each of these methods is well
25 represented in the art. Moreover, plasmids containing isolated polynucleotide sequences encoding CD40 receptor-associated factor polypeptide may placed into cells using many of these same methods.

30

35 CD40 receptor-associated factor itself may also be chemically modified to facilitate its delivery to a target cell. One such modification involves increasing

- 59 -

the lipophilicity of the CD40 receptor-associated factor in order to increase cell surface binding and stimulate non-specific endocytosis of the polypeptide. A wide variety of lipopeptides, fatty acids, and basic polymers (e.g., tripalmitoyl-S-glycerylcysteine-seryl-serine; palmitic acid; polyarginine) may be linked to an anti-fibrotic polypeptide to accomplish this. See U.S. Patent 5, 219,990, incorporated herein by reference.

Delivery may also be effected by using carrier moieties known to cross cell membranes. For example, an abnormal CD40 receptor-associated factor may be fused to a carrier moiety, preferably by genetic fusion, and the fused construct may be expressed in bacteria or yeast using standard techniques. Thus, polynucleotide sequences encoding abnormal or wild type CD40 receptor-associated factor useful in the present invention, operatively linked to regulatory sequences, may be constructed and introduced into appropriate expression systems using conventional recombinant DNA techniques. The resulting fusion protein may then be purified and tested for its capacity to enter intact target cells and inhibit growth of the target cells once inside the target. For example, recombinant methods may be used to attach a carrier moiety to anti-fibrotic polynucleotide sequences by joining the polynucleotide sequence encoding for abnormal CD40 receptor-associated factor with the polynucleotide sequence encoding a carrier moiety and introducing the resulting construct into a cell capable of expressing the conjugate. Two separate sequences may be synthesized, either by recombinant means or chemically, and subsequently joined using known methods. The entire conjugate may be chemically synthesized as a single amino acid sequence.

Useful carrier moieties include, for example, bacterial hemolysins or "blending agents" such as alamethicin or

- 60 -

sulfhydryl activated lysins. Other carrier moieties include cell entry components of bacterial toxins such as Pseudomonas exotoxin, tetanus toxin, ricin toxin and diphtheria toxin. Other useful carrier moieties include proteins which are viral receptors, cell receptors or cell ligands for specific receptors that are internalized and cross mammalian cell membranes via specific interaction with cell surface receptors. Such cell ligands include epidermal growth factor, fibroblast growth factor, transferrin and platelet derived growth factor. The carrier moiety may also include bacterial immunogens, parasitic immunogens, viral immunogens, immunoglobulins, and cytokines.

In one embodiment, purified human immunodeficiency virus type-1 (HIV) tat protein is the carrier moiety. Purified human immunodeficiency virus type-1 (HIV) tat protein is taken up from the surrounding medium by human cells growing in culture. See Frankel et al., *Cell* 55: 1189-1193, (1988); Fawell et al., *Proc. Natl. Acad. Sci. USA*, 91: 664-668 (1994) (use of tat conjugate); and Pepinsky et al., *DNA and Cell Biology*, 13: 1011-1019 (1994) (use of tat genetic fusion construct), all of which are incorporated herein by reference. See also PCT Application Serial Number PCT/US93/07833, published 3 March 1994 which describes the tat-mediated uptake of the papillomavirus E2 repressor; utilizing a fusion gene in which the HIV-1 tat gene is linked to the carboxy-terminal region of the E2 repressor open reading frame. The tat protein can deliver, for example, abnormal or wild type CD40 receptor-associated factor and polynucleotide sequences into cells, either in vitro or in vivo. For example, delivery can be carried out in vitro by adding a genetic fusion encoding an abnormal CD40 receptor-associated factor- tat conjugate to cultured cells to produce cells that synthesize the tat conjugate or by combining a sample (e.g., blood, bone

- 61 -

marrow, tumor cell) from an individual directly with the conjugate, under appropriate conditions. The target cells may be in vitro cells such as cultured animal cells, human cells or microorganisms. Delivery may be carried out in vivo by administering the CD40 receptor-associated factor and tat protein to an individual in which it is to be used. The target may be in vivo cells, i.e., cells composing the organs or tissue of living animals or humans, or microorganisms found in living animals or humans. The ADP ribosylation domain from *Pseudomonas* exotoxin ("PE") and pancreatic ribonuclease have been conjugated to tat to confirm cytoplasmic delivery of a protein. The ADP phosphorylation domain is incapable of entering cells so that cytoplasmic delivery of this molecule would be confirmed if cell death occurs. Likewise, ribonuclease itself is incapable of entering cells so that inhibition of protein synthesis would be a hallmark of intracellular delivery using a tat conjugate.

Chemical (i.e., non-recombinant) attachment of CD40 receptor-associated factor polypeptide sequences to a carrier moiety may be effected by any means which produces a link between the two components which can withstand the conditions used and which does not alter the function of either component. Many chemical cross-linking agents are known and may be used to join an abnormal or wild-type CD40 receptor-associated factor polynucleotide sequence or polypeptide to carrier moieties. Among the many intermolecular cross-linking agents are, for example, succinimidyl 3-(2-pyridyldithio)propionate (SPDP) or N, N'-(1,2-phenylene)bismaleimide are highly specific for sulfhydryl groups and form irreversible linkages; N, N'-ethylene-bis-(iodoacetamide) (specific for sulfhydryl); and 1,5-difluoro-2,4-dinitrobenzene (forming irreversible linkages with tyrosine and amino groups).

- 62 -

Other agents include p,p'-difluoro-m,m'-dinitrodiphenylsulfone (forming irreversible linkages with amino and phenolic groups); dimethyl adipimidate (specific for amino groups); hexamethylenediisocyanate (specific for amino groups); disdiazobenzidine (specific for tyrosine and histidine); succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC); m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); and succinimide 4-(p-maleimidophenyl)butyrate (SMPB). The succinimidyl group of these cross-linkers reacts with a primary amine, and the thiol-reactive maleimide reacts with the thiol of a cysteine residue. See, Means and Feeney, **Chemical Modification of Proteins**, Holden-Day, 39-43, 1974; and S.S. Wong, **Chemistry of Protein Conjugation and Cross-Linking**, CRC Press, 1971. All the cross-linking agents discussed herein are commercially available and detailed instructions for their use are available from the suppliers.

In clinical settings, the delivery systems for the abnormal or wild-type CD40 receptor-associated factor polynucleotide sequence can be introduced into a patient by any number of methods, each of which is familiar to persons of ordinary skill. Specific incorporation of the delivery system in the target cells occurs primarily from specificity of transfection provided by the gene delivery vehicle, cell type or tissue type expression due to the transcriptional regulatory sequences controlling expression of the polynucleotide, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being localized by, for example, catheter (U.S. Patent 5,328,470) or stereotactic injection (Chen et al., *Proc. Natl. Acad. Sci. USA*, 91: 3054-3057 (1994)).

Several methods of transferring potentially therapeutic

- 63 -

genes to defined cell populations are known. See, e.g., Mulligan, "The Basic Science of Gene Therapy", Science, 260, pp. 920-31 (1993). These methods include:

- 5 1) Direct gene transfer. See, e.g., Wolff et al., "Direct Gene transfer Into Mouse Muscle In Vivo", Science, 247, pp. 1465-68 (1990);
- 10 2) Liposome-mediated DNA transfer. See, e.g., Caplen et al., "Liposome-mediated CFTR Gene Transfer To The Nasal Epithelium of Patients With Cystic Fibrosis", Nature Med., 3, pp. 39-46 (1995); Crystal, "The Gene As A Drug", Nature Med., 1, pp. 16-17 (1995); Gao and Huang, "A Novel Cationic Lipoma Reagent For Efficient Transfection Of Mammalian Cells", Biochem. Biophys. Res. Comm., 179, pp. 280-85 (1991);
- 15 3) Retrovirus-mediated DNA transfer. See, e.g., Kav et al., "In Vivo Gene Therapy Of Hemophilia B: Sustained Partial Correction In Factor IX-Deficient Dogs", Science, 262, pp. 117-19 (1993); Anderson, "Human Gene Therapy", Science, 256, pp. 808-13 (1992);
- 20 4) DNA Virus-mediated DNA transfer. Such DNA viruses include adenoviruses (preferably Ad-2 or Ad-0 based vectors), herpes viruses (preferably herpes simplex virus based vectors), baculoviruses, and parvoviruses (preferably "defective" or non-autonomous parvovirus based vectors, more preferably adeno-associated virus based vectors, most preferably AAV-2 based vectors). See, e.g.
- 25 Ali, et al., "The Use Of DNA Viruses As Vectors For Gene Therapy", Gene Therapy, 1, pp. 367-84 (1994);
- 30 United States Patent 4,797,368, incorporated herein by reference, and United States Patent 5,139,941, incorporated herein by reference.
- 35

- 64 -

The choice of a particular vector system for transferring the gene of interest will depend on a variety of factors. One important factor is the nature of the target cell population. Although retroviral
5 vectors have been extensively studied and used in a number of gene therapy applications, these vectors are generally unsuited for infecting non-dividing cells. In addition, retroviruses have the potential for oncogenicity.

10 Adenoviruses can infect quiescent or terminally differentiated cells, such as neurons or hepatocytes, and appear essentially non-oncogenic. See, e.g., Ali et al., Supra, p. 367. Adenoviruses do not appear to
15 integrate into the host genome. Because they exist extrachromosomally, the risk of insertional mutagenesis is greatly reduced. Ali et al., Supra, p. 373. Adeno-associated viruses exhibit similar advantages as adenoviral-based vectors. However, AAVs exhibit site-specific integration on human chromosome 19. Ali et
20 al., supra, p. 377.

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery
25 system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is embedded. Where the complete gene delivery system can be produced intact from recombinant cells such as retroviral vectors, the pharmaceutical preparation can
30 include one or more cells which produce the gene delivery system.

Effective amounts of the compounds of the invention may be administered in any manner which is medically
35 acceptable. The method of administration may include injections, by parenteral routes such as intravenous, intravascular, intraarterial, subcutaneous,

- 65 -

intramuscular, intratumor, intraperitoneal, intraventricular, intraepidural, or others as well as oral, nasal, ophthalmic, rectal, topical, or inhaled. The term "pharmaceutically acceptable carrier" means one or more organic or inorganic ingredients, natural or synthetic, with which the molecule is combined to facilitate its application. A suitable carrier includes sterile saline although other aqueous and non-aqueous isotonic sterile solutions and sterile suspensions known to be pharmaceutically acceptable are known to those of ordinary skill in the art. In this regard, the term "carrier" encompasses liposomes or the HIV-1 tat protein (See Pepinsky et al., supra) as well as any plasmid and viral expression vectors. An "effective amount" refers to that amount which is capable of ameliorating or delaying progression of the diseased, degenerative or damaged condition. An effective amount can be determined on an individual basis and will be based, in part, on consideration of the symptoms to be treated and results sought. An effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

In preferred methods, an effective amount of the abnormal or wild-type CD40 receptor-associated factor (CRAF) or polynucleotide sequence encoding the factor (CRAF1 nucleic acid or a nucleic acid sequence encoding CRAF1 peptide) (contained within its attendant vector; i.e., "carrier") may be directly administered to a target cell or tissue via direct injection with a needle or via a catheter or other delivery tube placed into the cell or tissue. Dosages will depend primarily on factors such as the condition being treated, the selected polynucleotide, the age, weight, and health of the subject, and may thus vary among subjects. An effective amount for a human subject is believed to be in the range of about 0.1 ml to about 50 ml of saline solution

- 66 -

containing from about 1×10^7 to about 1×10^{11} plaque forming units (pfu)/ml CD40 receptor-associated factor polynucleotide (CRAF1 nucleic acid) containing, viral expression vectors. [U.S. Patent 4,363,877, Recombinant DNA Transfer Vectors, Goodman et al. is hereby incorporated in its entirety by reference.]

Target cells treated by abnormal or wild-type CD40 receptor-associated factor polynucleotide sequences may be administered topically, intraocularly, parenterally, intranasally, intratracheal, intrabronchially, intramuscularly, subcutaneously or by any other means. Target cells to be treated by abnormal or wild-type CD40 receptor-associated factor protein may be administered topically, intraocularly, parenterally, intranasally, intratracheal, intrabronchially, intramuscularly, subcutaneously or by any other means.

The protein compounds of the invention are administered at any dose per body weight and any dosage frequency which is medically acceptable. Acceptable dosage includes a range of between about 0.01 mg/kg and about 500 mg/kg subject body weight. A preferred dosage range is between about 1 and about 100 mg/kg. Particularly preferred is a dose of between about 1 and about 30 mg/kg. The dosage is repeated at intervals ranging from each day to every other month. One preferred dosing regime is to administer a compound of the invention daily for the first three days of treatment, after which the compound is administered every 3 weeks, with each administration being intravenously at about 5 or about 10 mg/kg body weight. Another preferred regime is to administer a compound of the invention daily intravenously at about 5 mg/kg body weight for the first three days of treatment, after which the compound is administered subcutaneously or intramuscularly every week at about 10 mg per subject.

- 67 -

The protein compounds of the invention, similarly to the therapeutic nucleotide sequences, may be delivered to tissues in a liposome-encapsulated formulation, or conjugated to carrier moieties such as HIV tat protein.

5 This delivery can be systemic, such as by intravascular delivery, or local. Local means of delivery of liposome-encapsulated compounds of the invention include intratumor or intraorgan injection. It also includes

10 local delivery by catheter, such as intrahepatic delivery into the portal vein, intrarenal or intraprostate delivery via the urethra, intracholecystic delivery via the bile duct, or delivery into various blood vessels of interest, particularly the coronary vessels or sites of vascular stenosis. Targeted

15 delivery may be accomplished by inserting components into the surface of the liposomes or other carrier moieties which confer target specificity. For example, areas of inflammation might be targeted by coating the carrier liposomes with monoclonal antibodies specific

20 for anti-CD40 ligand. Various types of tumors could be selectively targeted by coating liposomes with monoclonal antibodies specific for surface antigens characteristic of the tumor cells. Delivery of the novel polypeptides via liposomes may be particularly

25 advantageous because the liposome may be internalized by phagocytic cells in the treated animal, and because of improved stability. The liposome system may be any variety of unilamellar vesicles, multilamellar vesicles, or stable plurilamellar vesicles, and may be prepared

30 and administered according to methods well known to those of skill in the art, for example in accordance with the teachings of United States Patents 5,169,637, 4,762,915, 5,000,958 or 5,185,154, which are hereby incorporated by reference. In addition, it may be

35 desirable to express the novel polypeptides of this invention, as well as other selected polypeptides, as lipoproteins, in order to enhance their binding to

- 68 -

liposome.

The proteins of this invention may be used in the form of a pharmaceutically acceptable salt, suitable acids and bases which are capable of forming salts with the polypeptides of the present invention are well known to those of skill in the art, and include inorganic and organic acids and bases.

The compounds of the invention may be administered as a single dosage for certain indications such as preventing immune response to an antigen to which a subject is exposed for a brief time, such as an exogenous antigen administered on a single day of treatment. Examples of such an antigen would include coadministration of a compound of the invention along with a gene therapy vector, or a therapeutic agent such as an antigenic pharmaceutical or a blood product. In indications where antigen is chronically present, such as in controlling immune reaction to transplanted tissue or to chronically administered antigenic pharmaceuticals, the compounds of the invention are administered at intervals for as long a time as medically indicated, ranging from days or weeks to the life of the subject.

Example 2: Alteration of Expression of CRAF1-encoded Gene Products Are Associated with Hyper-IgM Syndrome (Low or Absent IgG, IgA, IgE): Target Validation for CRAF1 as a target for Immunosuppressive Therapy

Three individuals with non-X linked HIM (Patients A, B and C) that have normal CD40-L expression by the mAb 5c8 (and in the case of patient B by sequence analysis and normal expression of CD40 by the mAb G28-5 were analyzed. Both patients A and B are relatively healthy, since they are 49 and 27 years old, respectively, and have not developed neoplastic disease, nor has either

- 69 -

had pneumocystis, opportunistic infections or neutropenia, which are features of HIGMX-1 (37). Patient C is younger but is relatively healthy.

5 In vitro analysis of patient A and B's normal B cells showed that their isolated B cells are deficient at responding to anti-CD40 triggering (in the presence of rIL-4) because although these cells secrete large
10 amounts of IgM, they secrete low (barely detectable) amounts of IgG, IgA or IgE in vitro, whereas HIGMX-1 B cells are known to be induced to secrete IgG, A and E by α -CD40 (14-18,20,39). Patient C has not been evaluated in this assay. Together, these data further suggest
15 that B cell defects may be responsible for the HIM syndrome in patient A and B.

Since NH₂-terminal truncations in CRAF1 act as dominant negatives in cell culture (26), studies were undertaken to determine whether CRAF1 mutations are associated with
20 HIM in individuals with normal expression of CD40-L. Therefore, EBV-transformed lymphoblastoid cell lines were generated from the patients and patient C's mother. The B cell lines from these 3 unrelated patients with HIM (and the mother of patient C) were used to determine
25 the sequence of CRAF1 mRNA by RT-PCR. Sets of oligonucleotides designed to sequence CRAF1 were selected that amplified four overlapping regions of the CRAF1 cDNA.

30 Sequencing these cloned cDNA fragments revealed little evidence of PCR artifacts in these experiments, consistent with the fact that template mRNA (and later cDNA) was not limiting. The sequences revealed a polymorphism at nt 603 C→T in patients B and C and the
35 mother of C (but not in A), which alters codon 602-ACG->ATG and changes aa 128 T→M (which was also found in the cloning of CRAF1 by other groups (27,28).

- 70 -

Therefore, the PCR cloning and sequencing appeared to have high fidelity and not to have been the result of cross-contamination. From the DNA sequences cloned, consistent mutations were not identified that could relate CRAF1 defects to HIM. However, the lack of mutations suggested that CRAF1 splicing defects could alter signaling.

Analysis of CRAF1 peptides in HIM patients

Analysis of CRAF1 encoded peptides has recently become possible because of the availability of anti-CRAF1 antisera. Therefore, this antisera was used to determine if protein products of the potentially mutated alleles are present in cells from these individuals. A rabbit antiserum generated to the NH₂-terminus of the CRAF1 peptide was used to perform immunoprecipitation of metabolically labeled Ramos cells, Ramos/pCEP/CRAF1 and EBV-transformed B cell lines [pCEP is an expression vector available from Invitrogen]. Several bands are specifically immunoprecipitated by the anti-CRAF1 antisera, including major bands that migrate at 70 kDa and 60 kDa. The intensity of the 60 kDa band is strongly increased in lysates from Ramos/pCEP/CRAF1 cells, which suggests that this band represents the peptide product of the CRAF1-a transcript and which is consistent with the predicted molecular weight of a 568 aa peptide. The expression of both the 60 kDa band and the 70 kDa band is greatly increased in the EBV transformed cells relative to the intensity of these bands in Ramos controls suggesting that EBV transformation increases the expression of CRAF1 peptides and which is consistent with the observation that EBV upregulates CRAF1 mRNA in BJAB cells (27).

Immunoprecipitation of metabolically labelled EBV-transformed B cell lines from a normal individual and from the patients A and B was performed. In all three

- 71 -

cell lysates, anti-CRAF1 antisera immunoprecipitates major bands that migrate at 70 kDa and 60 kDa. (The intensity of the 60 kDa band is strongly increased in lysates from Ramos/pCEP/CRAF1 cells, which suggests that this band represents the peptide product of the CRAF1-a transcript.) However, this patient's B cell lysates contain a unique, approximately 77 kDa band (p70 or p77), that is not observed in the other EBV transformed cell lines or in the Ramos transfectants analyzed. Similar to the immunoprecipitates from patient B's cells, the lysates from the patient A cell line also contains the 60 kDa band, suggesting that if this patient has a mutated allele, a normal allele may also be present. It is unknown if the predicted 70 kDa peptide is relatively unstable or if some other process (potentially compensatory) is occurring in these cells, that would result in the 77 kDa (p70 or p77) band by a complex process, such as alternative splicing to delete a exon 3 that contains an in-frame termination codon. The Western blot data in Figure 5 suggests that p70 may not be rapidly degraded, since patient A has p70 peptide with a low rate of p70 synthesis.

Western blotting can be used to provide further evidence that the 30 kDa band that is specifically detected in patient B's cell lysates is a product of the CRAF1 gene or whether it represents a co-precipitating peptide that is unique to cells from this patient. (However, the fact that the 30 kDa band is predicted by the cloning data strongly suggests that this peptide does in fact, represent the CO₂H-terminal truncation.)

Characterization of the functional effects of inhibitory cDNAs of the p55 type in tumor cell models of CD40-triggering of B cells

To test whether carboxy terminal truncated proteins operate as dominant negatives in cultured cell lines,

- 72 -

stable transfectants of Ramos that express the 1-171 amino acids of p55 and the 1-258 amino acids of p55 were generated. Three clones of stable transfectants of both pCEP/CRAF1-PtA and pCEP/CRAF1-PtB in Ramos cells have been generated, and confirmed the presence of plasmid encoded CRAF1 mRNA has been confirmed by Northern analysis. As controls for this experiment, cDNA encoding full length 568 aa CRAF1 was cloned into the pCEP expression vector (Invitrogen) and expressed this clone in Ramos. The cell surface expression of CD23 and CD80 on Ramos/pCEP/CRAF1 transfectants shows that CD23 expression is not upregulated (relative to Ramos/pEBVHis/lacZ controls (Figure 3)). These data indicate that expression of full length CRAF1 does not induce the phenotype of a constitutively CD40-activated B cell, at least with respect to CD23 and CD80. Next, the responses of these transfectants to CD40 triggering was studied. In these experiments, CD40-triggering was induced by coculture with 293/CD40-L cells (or control 293/CD8 cells, not shown). In response to CD40 triggering with 293/CD40-L cells, Ramos/pCRAF1 cells have normal responsiveness with respect to upregulation of CD23 or CD80 or ICAM1. In functional experiments, the Ramos cells over-expressing the clones were triggered with 293/CD40-L⁺ cells and the expression of CD23 and CD80 was measured by two-color FACS. Interestingly, despite the modest expression of plasmid mRNA in these cells, the transfected clones were deficient at upregulating CD23 in response to stimulation with 293/CD40-L⁺ cells relative to control Ramos cells or Ramos cells expressing full length CRAF1. In this experiment, upregulation of CD80 by CD40 triggering was normal relative to control Ramos cells and to Ramos cells expressing full length CRAF1. The deficiency in CD23 upregulation has also been observed in non-transformed B cells from patients with non-CD40-L related HIGM (21). These data provide a molecular

- 73 -

mechanism by which CO₂H-truncated CRAF1 mutants result in HIM.

It can be determined whether the functional phenotype of these cells corresponds to the phenotype of the patients altered alleles expressed in Ramos cells as described above, however, it is interesting in this respect, that HIM patients with normal CD40-L have been described to have a deficiency in CD23 upregulation (37) and Patient B was one of the individuals in that study.

Characterization of the roles of CRAF1 in B cell signaling

Understanding of the role of CRAF1 in CD40 signalling has been extended by studying the functional deficit in Ramos cells stably expressing pEBVHis/C26 ("dominant negative") or pEBVHis/lacZ ("control") constructs. In experiments similar to those described for assessing CD23 upregulation, Ramos transfectants were co-cultured with 293/CD40-L cells or control 293/CD8 cells and the expression of CD80 (BB1-B7) and ICAM1 was measured by 2-color FACS. The CD40-L induced upregulation of CD80 (40) was substantially inhibited by C26 expression although this inhibition is not as potent as the effect of C26 expression on CD23. These data indicate that CD40-mediated upregulation of CD80 (which has been shown to correlate with the induction of co-stimulatory activity by B-CLL cells (see above)) appears to depend in large part on CRAF1 signaling. CD40 triggering also induces upregulation of CD54 (ICAM1) (40) and homotypic aggregation, but these effects are not inhibited by overexpression of the CRAF1 dominant negative C26-1 fusion protein. These data suggest that CD40 signaling involves at least one other component, or that the induction of ICAM1 upregulation is less sensitive to CRAF1 inhibition.

- 74 -

DISCUSSION:

These studies suggest an interpretation of CRAF1 signaling. The complete predicted aa sequences of human and mouse CRAF1 are highly homologous and reveal a Zn⁺⁺ ring domain, a Zn⁺⁺ finger domain, a coiled-coiled domain and importantly, that the -CO₂H-terminal domain that binds the CD40 cytoplasmic tail is homologous to two recently identified proteins, "TNF α Receptor-Associated Factors" 1 and 2 (TRAF-1 and TRAF-2) that form a complex with the cytoplasmic tail of TNFR α II (41). Thus CRAF1 peptide is a critical signaling factor in CD40 mediated responses and may transmit CD40 signals to the nucleus. More consistent with aggregation and less with proteolytic processing.

After the generation of stable transfectants expressing these constructs, the phenotype of the transfectants is analyzed (to determine whether constitutive activation of CD23 and/or CD80 expression is present) or CD40-triggered functional assays are performed to analyze the potential dominant negative effects of these constructs. Analysis of constitutive activation may be carried out by evaluating and detecting relative amounts of tumor cell death. One possible outcome of these experiments is that overexpression of the Zn ring and finger domains in isolation from other domains, will result in constitutive activation of Ramos cells (i.e., constitutive high expression of CD23 and CD80). This would be taken to suggest that CD40-signaling may activate CRAF1 by proteolysis which would liberate the DNA binding domain. Since the full length CRAF1 is not constitutively activating, this finding would suggest that other portions of the molecule (e.g., the coiled-coil domain) might normally retard the translocation of CRAF1 Zn ring/finger domains to the nucleus. This type of signaling has been described in the signaling of the sterol regulatory element-binding protein (SREBP-1)

- 75 -

(42).

Another possible outcome is that no domain is constitutively activating alone, but that, for example, the coiled-coil domain alone in a fusion protein, may function as a dominant negative (i.e., similarly to C26). These data would be consistent with a model in which CRAF1 signaling is primarily mediated by oligomerization of CRAF1 molecules, either to each other or in heteromeric complexes, this type of oligomerization is reminiscent of the JAK/STAT signaling system described by in interferon signaling (43). Moreover, this result would be interpreted in light of the results of experiments described below which ask whether CRAF1 is constitutively associated with CD40 or whether CD40 aggregation by trimeric CD40-L initiates the oligomerization of CRAF1 and potentially, whether such oligomerization of CRAF1 may result in its association with other signaling molecules, in analogy with the JAK/STAT system. Such results would also lead to re-interpretation of the mechanism by which the C26 peptide mediates its dominant negative activity. In this regard, how C26 mediates its dominant negative effect is under study. The simplest model to explain the dominant negative effects of C26 is that the C26-encoded fusion protein binds to the CD40 cytoplasmic tail and prevents the binding of full length CRAF molecules. However, the C-TRAF domain of C26 also mediates self-self oligomerization (26) and potentially, the C26 peptide may interfere with the oligomerization of endogenous CRAF1 molecules to mediate its dominant negative effects.

CD40 is a cell surface receptor for the T helper effector molecule, CD40-L (T-BAM, gp39, TRAP) and this interaction is essential for B cell selection and differentiation in vitro and in vivo. A protein that

- 76 -

binds to the cytoplasmic tail of CD40 termed "CD40 Receptor Associated Factor 1"(CRAF1) has been identified. Stable overexpression of the 240aa carboxy terminus of CRAF1 as a fusion protein (C26) in Ramos 2G6 B cells inhibits the CD40-dependent induction of CD23 expression (i.e. results in a functional "dominant negative"). To determine if CRAF1 is involved in other CD40 effector functions, Ramos B cells expressing the C26 fusion protein or control (expressing lacZ) were stimulated with anti-CD40 mAb (G28-5) and assessed for their ability to be rescued from Fas (CD95)-mediated apoptosis (Wright-Giemsa staining), to undergo homotypic aggregation (light microscope) or to upregulate CD54 (ICAM) or CD80 (B7/BB-1) (FACS). Similar to its effects on CD40-mediated upregulation of CD23, C26 inhibits anti-CD40 mediated rescue from Fas-mediated apoptosis and inhibits anti-CD40 mediated CD80 upregulation. However, C26 does not affect CD40-mediated CD54 upregulation nor homotypic aggregation, suggesting that the CD54 upregulation pathway is either less sensitive to C26 inhibition or possibly that CD40 has alternative (non-CRAF1) mediated signaling. Together, these data suggest that CRAF1 mediates the CD40 signals for rescue from apoptosis and upregulation of CD80, however, the CD40 signals for inducing ICAM are unresolved.

CD40-L is known to have a different role from CD95 in germinal center biology because mutations in CD40-L impair germinal center formation as manifest in humans that have absent or mutant CD40-L protein expression (X-linked, hyper-IgM syndrome (HIGMX-1) or mice with targeted disruptions of CD40-L or CD40. The lack of germinal centers in HIGMX-1 is associated with extensive IgM+ B cell infiltration of tissues, particularly in the GI tract (11,13-18,20), which may relate to the predisposition of HIGMX-1 patients to develop polyclonal B cell pseudolymphomas (11,13). These data suggest that

- 77 -

apoptosis in germinal centers play important roles in limiting IgM⁺ B cells and to the extent that CD95 represents a limiting step in mediating B cell apoptosis in germinal centers, these data suggest that CD95 normally regulates B cell homeostasis and limits B cell transformation. Although the role of CD40-L in germinal center formation is elucidated by the lack of germinal centers in HIGM1-1, this syndrome does not address the role of CD40-L on T cells in germinal centers.

EXPERIMENTAL MATERIALS AND PROCEDURES

Monoclonal Antibodies

The mAb 5c8 (anti-CD40-L) has been described (1). The following mAb were produced from hybridomas available from the American Type Culture Collection (Rockville, MD): OKT8 (anti-CD8), DA4-4 (anti-IgM) and W6/32 (anti-MHC class I). These mAb were purified from ascites fluid on protein A (Biorad, Rockville Center, NY) or protein G columns (Pharmacia, Upsala, Sweden). Leu-16 (Anti-CD20) mAb conjugated to fluorescein and Leu-17 (Anti-CD38) mAb conjugated to PE were purchased from Becton Dickinson (Mountainview, CA). BB20 (anti-CD40) was purchased from Biosource International (Camarillo, CA).

Cell Lines

The human B cell lymphoma clone RAMOS 2G6.4CN 3F10 (Ramos 2G6) is available from ATCC. 293/CD8 is a human kidney tumor cell line that is stably transfected with CD8 as described (10,50). Stable CD40-L⁺ clones of 293 cells (293/CD40-L) were generated by electroporation of a cDNA that encodes CD40-L (pCD40-L/SpeI) and cloning of transfectants that express surface CD40-L by mAb 5c8 binding. To generate pCD40-L/SpeI, a CD40-L cDNA (pTBAM described in Covey et al. (10)) was used as template for PCR amplification using a 5'-oligonucleotide (oCD40-L/SpeI: 5'-GCA gCT AGC CAC AGC ATG

- 78 -

ATC GAA ACA TAC AAC CAA ACT AGT CCC CGA TCT GCG-3') (Seq I.D. No. ____) that altered the CD40-L sequence of the amplified product to create a **Spe I** site at position 25, a **Nhe I** site at position -12, and to append the putative 5'-UT Kozak sequence (6). The 3' oligonucleotide corresponds to pCCD40-L 616-633 (oCD40-L⁶³³⁻⁶¹⁶: 5'-CTT TAG GCA GAG GCT GGC-3') (Seq I.D. No. ____). The PCR product generated was digested with **Nhe I** and **Hind III**, cloned into the expression vector pREP4 (Invitrogen, San Diego, CA) and its sequence was confirmed by automated sequencing. The CD40-L coding region was completed by insertion of the 3' end of the pTBAM cDNA as a **Hind III** -**Not I** fragment from pCCD40-L, yielding pCD40-L/**Spe I**.

Isolation of Tonsillar B lymphocytes

Tonsil B cells were obtained from fresh surgical specimens after tonsillectomy. The lymphoid tissue B cells were obtained by mincing tissue specimens and passing them through a metal screen followed by ficoll-hypaque (Sigma, St. Louis, MO) centrifugation. B cells were derived from the population of cells that did not pellet through Ficoll-Hypaque after one round of rosetting with neuraminidase-treated sheep erythrocytes. Tonsillar B cells were further purified by density centrifugation. Cells were fractionated into high (resting) and low (germinal center CD38 enriched) density cells in a discontinuous 30%/50%/100% percoll gradient by centrifugation at 2300 rpm for 12 min. High density cells were obtained from the 50%/100% interface and low density cells from the 30%/50% interface (33,51).

Cytofluorographic analysis of Tonsillar B cells

Approximately 10^5 cells were incubated with saturating concentrations of the indicated mAbs for 45 min at 4°C in the presence of 80 µg/ml heat aggregated IgG (International Enzyme, Fallbrook, CA). Cells were

- 79 -

washed to remove unbound antibody before incubation with F(ab)₂ goat anti-mouse Ig secondary antibody coupled to fluorescein (Jackson ImmunoResearch, Westgrove, PA). Two color **FACS** (Fluorescence Assisted Cell Sorter) analysis involving both direct and indirect antibody staining was accomplished as above for indirect antibody staining except that cells were washed prior to incubation with conjugated antibody. Fluorescence intensity was measured on a FACScan cytofluorograph (Becton Dickinson) using Consort 30 software.

Assays of Anti-IgM and anti-CD95 induced apoptosis

Ramos B cells (3×10^6) or freshly isolated low density or high density tonsillar B cells (5×10^6) were added to each well of sterile 12 well tissue culture plates (Costar, Cambridge, MA) and incubated with media alone 0.5 $\mu\text{g/ml}$ anti-CD95 mAb (APO-1/huFc γ 1) or control chimeric anti-CD4 (mAb M-T412/huFc γ 1), 5 $\mu\text{g/ml}$ anti-IgM or 5 $\mu\text{g/ml}$ or 0.5 $\mu\text{g/ml}$ of control anti-Class I (mAb W6/32) as indicated in figure legends. In addition, wells contained either media, 0.5×10^6 TBAM/293 cells or CD8/293 cells in the presence or absence of mAbs anti-CD40-L (5c8) or control mAb (W6/32) in a final volume of 1 or 2 ml. Cultures were incubated for either 10h or 14h (anti-CD95 induced apoptosis) or 36 h (anti-IgM induced apoptosis) before cells were harvested and analyzed for apoptosis by cell morphology and DNA fragmentation into nucleosomal units.

Determination of Apoptosis by Cell Morphology

Low density tonsillar B cells or Ramos 2G6 cells were treated as indicated above. Cells were washed and 1×10^5 were cytopspun onto microscope slides using a Cytospin 3 (Shandon, Cheshire England). Cells were stained for two minutes with Wright-Giemsa (Sigma, St. Louis, MO), rinsed with distilled water and dried before observation by light microscopy (Olympus CK2). Cells were scored as

- 80 -

viable (non-apoptotic) if they maintained an intact cell membrane and nucleus and did not display membrane blebbing, condensed cytoplasm, pyknotic nuclei or other characteristics of an apoptotic cell(52). Photographs of representative cell populations were taken by an Olympus OM-2S camera.

Determination of Apoptosis by DNA fragmentation and Cell Viability

DNA fragmentation was assessed by a modification of Benhamou et al. (53). Treated cells were washed twice in HBSS and lysed in 400 μ l of buffer containing 10 mM EDTA, 200 mM NaCl, 0.1 mg/ml Proteinase K, 0.5% SDS, and 50 mM Tris-HCl (pH 8.0). After 1h incubation at 50°C, lysed cells were subjected to phenol-chloroform extraction. Genomic DNA was precipitated by the addition of 2 volumes of 100% ethanol and was removed using a sterile pipette tip. Fragmented DNA was precipitated after the addition of 1/10 volume of 3 M NaOAc (pH 7.2) and incubation at -70°C for 2-5 h. Precipitated DNA was then washed with 70% ethanol, dried, and resuspended in 20 μ l of RNase buffer consisting of 15 mM NaCl, 10 mM Tris-HCl (pH 7.5) and 5 μ g/ml RNase A. After incubation at 50°C for 1 h, DNA was electrophoresed on a 1.5% agarose gel at 80 V for 1-3 h and stained with ethidium bromide. This method allows for the isolation of fragmented DNA only and therefore can be used to assess the relative level of apoptosis in the B cell population when total sample is loaded to each well, since each sample contains an equivalent number of tonsillar or Ramos B cells and 293 cells are resistant to CD95 induced apoptosis.

Primers for PCR

The oligonucleotide primers used in these experiments are designated "o", followed by "CR" for CRAF1, followed by "f" or "r" for forward and reverse

- 81 -

respectively and then the nucleotides that they correspond to with the numbering of the CRAF1 nucleotide sequence of clone IIIb that is deposited in Genbank U21092). These oligos include: oCRf(127-144) (5'-AgC AgA ACg CTg Cgg ACC) (Seq. I.D. No. ____), oCRr(675-658) (5'-CAG TCA ggA CgC ACA CAT) (Seq. I.D. No. ____), oCRf(486-503) (5'-CgT gTC AAg AgA gCA TCg) (Seq. I.D. No. ____), oCRr(1091-1074) (5'-gCA AAC TTT gTA TgC TCT) (Seq. I.D. No. ____), oCRf(902-919) (5'-CCC AgC ACC TgT AgT TTT) (Seq. I.D. No. ____), oCRr(1450-1433) (5'-ggT CTC CAg gAC CTg gAA) (Seq. I.D. No. ____), oCRf(1164-1181) (5'-TCC TTC ATT TAC AgC gAg) (Seq. I.D. No. ____), oCRr(2119-2102) (5'-gAA gTg Tgg CTA gTC TAT) (Seq. I.D. No. ____), oCRf(550-567) (5'-ggC TCT TCA gAT CTA Ttg) (Seq. I.D. No. ____), oCRr(144-126) (5'-ggT CCg CAg CgT TCT gCT C) (Seq. I.D. No. ____), oCRr(213-197) (5'-ggA AAg Agg AgT TCT Cg) (Seq. I.D. No. ____) and oCRr(289-272) (5'-gTC AgT gTg CAg CTT TA) (Seq. I.D. No. ____).

The four overlapping cDNA fragments were amplified by RT-PCR in which the RT reaction was primed with oCRr(2119-2102). The following pairs were found to produce the expected products in RT-PCR on mRNA from a control EBV transformed B cell line: including (1.) oCRf(127-144)/oCRr(675-658) yields product (127-675), (2.) oCRf(486-503)/oCRr(1091-1074) yields product (486-1091), (3.) oCRf(902-919)/oCRr(1450-1433) yields product (902-1450) and (4.) oCRf(1164-1181)/oCRr(2119-2102) yields product (1164-2119). The four overlapping regions in each of the patients (including those of the mother of patient C) were amplified, the amplified fragments were isolated and cloned in the TA cloning system (pCRII, Invitrogen) and the inserts from these plasmids were sequenced in both directions.

To generate cDNAs containing the mutations from patient A and B, the PCR product generated by oCRf(550-567) and

- 82 -

oCRR(1091-1074), which yields product (550-1091) was cloned into pCRII (Invitrogen) and digested with *Bgl* II (which cuts within oCRf(550-567)) and *Eco*RI (which cuts in the polylinker of pCR-II and generates similar fragments (except for the patient A or B sequence transitions) independently of the fact that the PCR products from A and B were initially cloned into pCRII in different orientations. The *Bgl*III/*Eco*RI fragments of patient cDNAs were ligated into pCRAF1/Bluescript digested with *Bgl* II/*Eco* RI to yield fragments that contain the premature termination codons of patient A and B. The cDNAs encoding the full coding sequence of the patients were then digested with *Not* I/*Xho* I and ligated into pCEP/CRAF1 and pEBV/His/CRAF1.

Example 3: CRAF-1 Isoforms with Zinc Finger Deletions

One isoform of CRAF-1 peptide is a 568 aa signaling protein that interacts with the cytoplasmic tail of B cell surface molecule CD40 and mediates a variety of T-dependent effects on B cell activation and differentiation. CAP-1, which is a cDNA related to CRAF-1 nucleic acid, is similar to a CRAF-1 nucleic acid except for a 75 nucleotide deletion in a region which, in a CRAF-1 nucleic acid embodiment provided for herein, encodes five zinc finger-like domains. To determine whether alternative mRNA splicing from a common CRAF-1 gene accounts for this difference, the structure of human CRAF-1 nucleic acid mRNA and genomic DNA was analyzed using RT-PCR (reverse transcriptase - PCR) and long-template PCR amplification, respectively, followed by subcloning and sequence analysis. In addition to a CRAF-1 nucleic acid and CAP-1(del aa218-242) encoding transcripts, a third mRNA species was identified that predicts a peptide with a larger (52 aa) deletion in this region (del aa191-242). Analysis of the genomic structure of a CRAF-1 gene in this region identified exons that encode aa191-217 and aa218-242. These exons

- 83 -

are separated from each other by a 3.5 kB intron and from the adjacent 5'- and 3'- exons by 9.5 kB and 3.5 kB introns, respectively. The loss of these exons in the two smaller transcripts suggests that these deletions arise by alternative mRNA splicing. The location of these deletions within the zinc finger-like region of CRAF-1 nucleic acid predicts the loss of two or three zinc fingers, in the CAP-1(del aa218-242) and del aa191-242 forms, respectively.

In other proteins which contain zinc finger domains, such zinc finger domains interact with other proteins, RNA or DNA. Together, these data suggest that differential splicing modulates CRAF-1 peptide signaling by generating a variety of CRAF-1 peptide isoforms which differentially interact with other constituents of the cytoplasmic signaling apparatus.

Enhancing Alternative Splicing

Because alternative splicing removal of particular exons results in inhibitory CRAF1 isoforms, agents that drive such alternative splicing are expected to have beneficial effects similar to mAb 5c8 treatment (See U.S. Patent No. 5,474,771, issued December 12, 1995). Such agents include gene therapy constructs that encode elements of the splicing apparatus. These constructs may be used singly or in combination. For example, certain elements of the splicing complex that have effects on RNA splicing generally, may be over-expressed alone, or in combinations with other elements of the splicing complex that has specific targeting effects on the CRAF-1 RNA splicing. These elements include both gene therapy constructs that encode protein elements of the splicing complex as well as others that encode RNA elements of the splicing complex. Activity of gene therapy constructs comprising the whole proteins or whole RNA molecules that participate in RNA splicing

- 84 -

allows the identification of smaller constructs containing minimal essential elements of these nucleotide segments, for example steroid-like molecules. Furthermore, the identification of the minimal elements leads to the identification of smaller organic molecules that have the essential chemical features and biological activity of the larger DNA elements (enhancing the RNA splicing that generates CRAF-1 isoforms by deleting a particular the RNA of a particular exon).

Such agents include preferentially target the splicing of the particular exon which is removed. Such agents include cytokines and small organic agents that induce cell differentiation or activation.

Specific examples of elements of the splicing apparatus which can be used to enhance splicing in the method of this invention include small nuclear RNAs (snRNAs) such as U1, U2, U3, U4, U5 and U6. Because these snRNAs exist stably complexed with certain nuclear proteins, splicing can also be enhanced using small nuclear ribonucleoproteins (snRNPs). (See Watson, et al. Molecular Biology of the Gene, 4th ed. (Benjamin/Cummings: Menlo Park 1987) pp. 640-644.). A number of splicing factors or enhancers of pre-RNA splicing have been identified in Drosophila, including Transformer 1 (Tra1), Transformer 2 (Tra2), SF2/ASF, SC35, Srp20, Srp55, Srp75, U2AF⁶⁵, and U2AF³⁵. (See Amrein, et al. Cell (1994) 76:735-746; Heinrichs, et al. EMBO J. (1995) 14: 3987-4000; Lynch et al., Genes & Development (1995) 9:284-293). Factors which enhance RNA splicing generally, or CRAF1-specific splicing are used to enhance splicing in the zinc-finger region of CRAF1.

Example 4: Chromosomal Localization, Genomic Structure, And Identification of Alternatively Spiced mRNAs Of

- 85 -

Human TRAF-3

TRAF-3 (CRAF-1, LAP-1, CD40bp) is a cytoplasmic signaling molecule that interacts with the cytoplasmic tail of CD40 and transduces contact dependent signals for B cell activation. The gene for TRAF-3 was characterized by FISH (fluorescence in situ hybridization) and by radiation hybrid mapping and found to be located at chromosome 14q32.2, approximately 1 Mbase centromeric to the Ig heavy chain locus. TRAF-3 was further characterized by genomic PCR and sequencing genomic λ -phage and P1 clones. TRAF-3 is encoded by 10 exons which roughly correspond to protein domains predicted by the TRAF-3 cDNA. Interestingly, a variety of TRAF-3 cDNA species were identified which are generated by alternative mRNA splicing of distinct exons. Many of these species predict novel TRAF-3 peptides which vary in critical structural regions of this signaling protein.

INTRODUCTION

TRAF-3 (CRAF-1, LAP1, CD40bp) is a 568 amino acid cytoplasmic signaling molecule that plays an important role in the transduction of signals from the CD40 receptor in B cells. TRAF-3 has also been shown to bind the cytoplasmic domain of at least one other membrane receptor, the receptor for lymphotoxin- β , and TRAF-3 mRNA is expressed by cells of many lineages. Furthermore, in addition to physiologic signaling, TRAF-3 may also play a role in EBV-driven B cell proliferation and immortalization, by binding EBV latent membrane protein-1.

TRAF-3 is a member of the TNF α -receptor associated factor family "TRAF family" of signal transducing molecules on the basis of homology in the carboxy terminus of family members TRAF-1 and TRAF2. These

- 86 -

signaling molecules contain a COOH-terminal TRAF subdomain (TRAF-C) that mediates protein:receptor binding. In addition to the TRAF-C domain, sequence analysis of the TRAF-3 protein has revealed four other potential domains, including a zinc ring, a complex composed of five atypical zinc fingers, a coiled-coil (isoleucine zipper) structure, and an NH3-terminal (TRAF-N) domain. In other molecules, zinc rings, zinc fingers, and coiled-coil structures mediate protein-protein interactions. These structural data suggest interactions by which TRAF-3 may transmit CD40 signals, for example by interacting with other cytoplasmic proteins, including other TRAF-3 molecules or TRAF family members. Therefore, the distinct structural domains of TRAF-3 may play distinctive roles in TRAF-3 function.

Interestingly, several lines of evidence have suggested that these domains may be differentially expressed in different TRAF-3 isoforms. For example, in the deposited sequences for murine and human TRAF-3, there are nucleotide sequences which are related by the inclusion or exclusion of distinct gene elements, consistent with alternative splicing, in the portion of the TRAF-3 cDNA that encodes 5'-UTR (untranslated region) DNA. Furthermore, one TRAF-3 species (called IIIb) contains an open reading frame that extends upstream from the start site of 568 aa TRAF-3. Another species of TRAF-3 was reported by Reed, and termed CAP-1, which predicts a peptide with a 25 aa deletion in the zinc finger complex.

The chromosomal localization and genomic structure of TRAF-3 was determined, in order to understand the structure and genesis of TRAF-3 mRNA. Various isoforms of TRAF-3 were identified which suggest that alternative splicing of TRAF-3 mRNA may generate distinct TRAF-3

- 87 -

peptides. Such isoforms may play different roles in the signaling function of TRAF-3, and may thus represent a mechanism for the regulation of TRAF-3 in B cells.

5 CHROMOSOMAL LOCALIZATION OF TRAF-3

To identify the chromosomal localization of TRAF-3, fluorescence in situ hybridization (FISH) analysis of human fibroblast metaphase spreads was undertaken using genomic λ phage clone #6 as a probe. This probe bound specifically to the telomere of the long arm of human chromosome 14, at position 14q32.

Since this region is known to contain the human immunoglobulin heavy chain locus, and since this locus contains a well-characterized breakpoint in Ramos cells, a non-EBV infected Burkitt's tumor cell line, localization of TRAF-3 with respect to this chromosomal landmark was performed. Two-color FISH was performed on metaphase chromosomes from Ramos cells using the TRAF-3 probe and a chromosome 14 telomere probe. The two probes bound in very close proximity on the normal chromatid, within an estimated distance of 1 Mb. Only the TRAF-3 probe, however, bound to the homologue of chromosome 14 that had undergone breakage, whereas the chromosome 14 telomere probe bound to chromosome 8, consistent with the translocation of this telomere to chromosome 8 in Ramos.

Taken together, these data indicate that the TRAF-3 gene is located centromeric to the Ramos breakpoint on 14q32. The close physical proximity of the two probes further suggested that TRAF-3 and this breakpoint, [which is within the C μ switch region in the immunoglobulin heavy chain locus] are located within one Mb of each other, an estimate that was confirmed by a PCR-based screening assay of a somatic cell hybrid panel. From this screen, the TRAF-3 locus lies within 3.5 cR (approximately 1 Mb)

- 88 -

of framework markers FB19F11 and WI-9179 on the Whitehead radiation hybrid database.

CHARACTERIZATION OF THE GENOMIC STRUCTURE OF TRAF-3

5 Isolation and Characterization of TRAF-3 cDNA clones

A Raji cDNA library was screened with C26, a cDNA probe that represents the terminal 1271 nt of the TRAF-3 open reading frame. A second screen was performed with NotI-SacI, a probe which represents the first 447 bp of IIIb. 10 These screens revealed unexpected diversity in the 5' portion of TRAF-3 when compared with IIIb, the full-length TRAF-3 sequence which was initially cloned from this library. IIIb contains 206 bp of the TRAF-3 5'-untranslated region (called exon 3) as well as the 15 entire TRAF-III open reading frame. One clone, Ib, was identical to IIIb except for the inclusion of an 139 bp exon, which was called exon 3. This exon contains a stop codon in the reading frame of exon 4 which contains the p55 translational start site (IIIb nt 224; Figures 20 1A-1P, nucleotide 679). Another clone, 2-1, contained exon 3, and a third unique exon, at least 77 bp long, which precedes exon 3, and was called the product of exon 1. Several lines of evidence suggest that these diverse mRNA species were represented in mRNA and not 25 artifacts of the cloning procedure. RT-PCR, for example, revealed the appropriate products encoding IIIb-like, Ib-like and 2-1-like species. Moreover, 5'-RACE (Rapid Amplification of cDNA Ends) revealed the identical arrangement of exons, but did not reveal 30 upstream sequences that were distinct from those contained in the characterized cDNA clones. These data suggested that alternative splicing of distinct exons resulted in the 5' variation seen in these three TRAF-3 cDNA clones.

35

Isolation and Characterization of TRAF-3 Genomic clones

From a human genomic phage library (referred to P1 or as

- 89 -

pAC clones) three clones (#4, #6, and #9) that hybridized with cDNA probe HincII-HincII (IIIb 606-1003) were isolated. These clones were characterized by restriction digestion with [NotI and EcoRI] and by PCR mapping. Each phage clone contained an insert of about 20 kb. Two of these clones (#4 and #9) were found to be overlapping but distinct, containing the 3' end of the gene [nt 981-2437 on IIIb cDNA]; the third clone, #6, was found to encode the 5' end of the gene [nt 207-793 on IIIb cDNA]. The gap from nt 794-981 is covered by pAC clone #167.

To further characterize the genomic structure of TRAF-3, a human genomic P1 (or pAC) artificial chromosome library was screened with two different TRAF-3 genomic probes, ff7gen-fr8gen and pst1c-r2g1x. A 124 kb P1 clone (pAC clone #167) (ATCC Accession No. 97962) that hybridized to both probes was isolated and characterized by genomic PCR. This clone was found to contain DNA that encoded the entire open reading frame of human TRAF-3.

Linking 5'-UTR to the TRAF-3 coding region

The TRAF-3 5'-UTR was not contained on the most 5' λ -fix clone (clone #6) or on P1 clone #167 by Southern blotting with probe CE, a 193 bp fragment which contains part of the TRAF-3 5'-UTR including exon 3. Therefore, the genomic library was re-screened with this probe, and a single P1 clone [#34] (pAC clone #34, ATCC Accession No. 97963) was isolated. Restriction digestion of P1 clone #34 with EcoRI identified a 3.8 kb genomic restriction fragment that hybridized to the CE probe. Furthermore, a 6.0 kb EcoRI fragment was identified that hybridized to zfl1, a 25 bp end-labeled oligonucleotide primer that represents part of exon 1. A third EcoRI fragment, 7.0 kb long, hybridized to probe NotI-SmaI, which represents the 5' half of exon 2. All three

- 90 -

genomic fragments were subcloned into Bluescript and sequenced in both directions, using exon-specific primers, confirming these data.

5 Although these studies identified genomic DNA that encodes the 5'-UTR, none of these clones hybridized with probes that contained the coding region of TRAF-3. Genomic PCR between exon 3 and the TRAF-3 coding region using uncloned DNA as a template did not generate a
10 reaction product. Similarly, genomic PCR between each of the exons in the 5'-UTR, using both uncloned DNA and P1 clone #34 as template, did not generate a reaction product. It is therefore estimated that the separation between exon 3 and exon 4 (which contains the start site
15 of p55) between the exons is at least 28 kilobases. The separation between exon 2 and exon 3 is about 54.5 kb.

The CRAF exons were flanked on the 5'-side of ;AC #34 by ORF (D14S72) and on the 3'-side of pAC #167 by EST (SGC
20 30775), confirming the chromosomal location determined by FISH and radiation hybrid mapping and which indicated that pAC #34 is centromeric to pAC #167 and that the transcriptional orientation of the CRAF1 gene is centromeric to telomeric, or in the reverse orientation
25 of the Ig heavy chain gene locus (transcriptional orientations are indicated by arrows in Figure 6.

Establishing exon/intron boundaries

Relevant regions of genomic clones (λ fix clones #6 and
30 #9 and pAC clones #167 and #34) were sequenced in both directions using oligonucleotide primers developed from TRAF-3 cDNA clones Ib, IIIb, and 2-1 [Tables 1 and 3]. Comparison between genomic and cDNA sequence data permitted the definition of exon/intron boundaries for
35 each of the TRAF-3 (p55) coding exons as well as the 3' borders of exons 1, 2 and 3. The 3' border of exon 1 was determined from the spliced species which

- 91 -

represented an exon 1,3 product. However, the 5' border of exon 2 could not be established from cDNA's obtained, which did not include an example of an exon 1, 2, 4 product, presumably because of the high G-C content of this region. Therefore, the 5' border of exon 2 was deduced by consideration of the potential splice donor sites in this region, only one predicted an ORF as a putative 1,2,4 product. Therefore, 5'-boundary of exon 2 shown in Figures 1A-1P is the only border that would encode a novel amino-terminal domain. Forward intron sequencing primers, 50-100 bp upstream of each exon, which were used to confirm these data [Table 3]. The TRAF-3 (p55) coding sequence is composed of 10 exons, which follow consensus splice donor and acceptor rules for exon/intron boundaries, and which were called 4 to 13. (See also Figures 1A-1P.)

Establishing intron size in TRAF-3 coding region

Determination of exon/intron boundaries allowed use of a PCR-based approach in order to determine intron size. PCR was performed on both control human genomic DNA and on λ -phage and P1 genomic clones. Oligonucleotide primer pairs spanning each of 9 consecutive coding exon/intron junctions were developed from the IIIb cDNA sequence. These were used to amplify genomic human DNA. The resulting PCR products, consisting of an intron bordered by two adjacent exon fragments, were sequenced to confirm their identity. The nine introns identified in this way ranged in size from 600 bp to 9.5 kb [Table 3].

ALTERNATIVE SPLICING GENERATES DIFFERENT TRAF-3 ISOFORMS

A variety of EBV-transformed B cell lines were obtained from HIGM patients A, C, and B, and unaffected relatives of HIGM patients CM and DM. RT-PCR amplification of human TRAF-3 mRNA from these five donors revealed subtle length variations in cDNAs amplified from two regions of

- 92 -

TRAF-3: the 5'-UTR and the zinc finger region. In all cases examined these length variations corresponded to the alternative splicing of specific TRAF-3 exons.

5 **Alternative splicing of exon 3**

Primers df7 and fr8, which span part of the 5'-UTR and the first 457 bp of the TRAF-3 coding sequence, were used to amplify cDNA from subjects A, B, C, and CM [Table 3]. These primers generated two TRAF-3
10 amplification products in approximately equal amounts. The first was the 548 bp predicted from the published TRAF-3 sequence. The second was a 687 bp fragment, which was detected in three of the four subjects studied [B,A,CM], and which was found to be identical to the
15 smaller fragment except for the insertion of exon 3 in the 5'-UTR.

Zinc finger variants

External primers 3b210f and 3b1930r, which span the
20 entire 1.9 kb TRAF-3 coding sequence, were used to amplify cDNA from subjects CM and DM. Sequencing of these variant amplification products revealed deletions of 75 bp (IIIb nt 875-949), 156 bp (IIIb nt 794-949), and 168 bp (IIIb nt 875-1042) in the zinc finger region.
25 These deletions corresponded to the alternatively spliced loss of exons 9 (identified by Sato et al as CAP-1); loss of exons 8 and 9; and loss of exons 9 and 10. Such splicing is apparently uncommon, as the predominant isoform was the full length one, and only
30 rare subclones contained the shorter, internally deleted messages.

DISCUSSION

Results of chromosomal FISH analysis and radiation
35 hybrid screening in this study suggest that TRAF-3 is a single copy gene located on chromosome 14q32.2. This region plays a major role in B cell development as the

- 93 -

immunoglobulin heavy chain switch region, which normally undergoes germline rearrangements during B cell development, and which is frequently involved in various translocations in certain hematologic diseases. It is therefore possible and even likely that the expression of TRAF-3 is regulated by the genomic splicing that takes place at this locus during normal B cell development, or is dysregulated by translocations. What the results of such events might be are difficult to predict. It may be for example, that normal rearrangements regulate expression of TRAF-3 by inactivating one allele, or that TRAF-3 is upregulated or, like *bcr-abl*, fused with other gene products during rearrangement or translocation.

Furthermore, the chromosomal localization of TRAF-3 may play a role in identifying that subset of HIGM patients who have a TRAF-3 defect. While the essential roles of CD40-L and CD40 in the phenotype of HIGM syndrome has been confirmed by targeted disruption of either CD40-L or CD40 in mice, no such evidence has yet been found for that subset of non-X-linked HIGM patients. Determination of defects in TRAF-3 may mediate immune deficiency syndromes with the HIGM phenotype. A subset of individuals with HIGM syndrome have normal CD40-L.

TRAF-3 may be linked with other genes that are associated with human diseases. In this regard, a form of HIGM with normal CD40-L was described that is associated with hypohidrotic ectodermal dysplasia, but in this family the syndrome appears to be inherited in an X-linked pattern and therefore may represent a different form of non-CD40-L HIGM. The chromosomal localization of TRAF-3 will be of interest, therefore, in identifying this and related syndromes.

Structural analysis reveals that TRAF-3 p55 is encoded

- 94 -

by 10 exons (exons 4-13), which correspond roughly to protein domains predicted by the TRAF-3 cDNA sequence. Thus, exons 4 and 5 encode the zinc ring, exons 6-10 encode the zinc finger complex, exon 11 and the NH₂-terminal encoding half of exon 12 encode the helical wheel, and the remaining half of 12 and 13 encode the N-TRAF domain and the TRAF-C domain is encoded by exon 13. Such structural detail is of general interest, as none of the TRAF family members have until now been analyzed at this level, and given their homology and similar functions, they presumably all have similar exonic arrangements. At the genomic level, for example, most of the structural complexity in TRAF-3 is seen in the zinc finger complex, a domain which appears to be regulated by alternative splicing. Interestingly, it has been noted that TRAF-2, which also has a multi-zinc finger domain, appears to undergo similar splicing.

The zinc finger complex consists of a single large exon, which encodes the 5' half of this region followed by three smaller exons, which encode the remaining zinc fingers. The organization described here suggests that the TRAF-3 zinc finger complex has evolved by the duplication of an original progenitor exon, leading to the addition of successive zinc fingers. Interestingly, a comparative amino acid analysis of TRAF-3 in the zinc finger complex shows that in the 3' half of this domain there is 100% identity between murine and human sequences. By contrast, seven of the 80 preceding amino acids that make up the 5' half of this complex are divergent, and four of these seven changes are non-conservative. Such conservation in the 3' part of this complex implies that this portion of the zinc finger complex is significant in the signaling function of TRAF-3.

A total of three unique mRNAs which vary in this region

- 95 -

have been isolated, the deletion of exon 9, deletion of exons 8 and 9, and deletion of exons 9 and 10 as TRAF-3 isoforms. Each of these variants is in frame, and each deletion corresponds to the loss of one or two adjacent exons in this region. Therefore, each isoform encodes a different combination of zinc fingers in the 3' half of this complex. This interchangeability of exons is made possible by their common coding class, which ensures that removal or insertion of a given exon will not affect reading frame [Table 3]. The possible consequences of such splicing are intriguing, in that zinc fingers commonly mediate both protein-protein and protein-DNA interactions. Each of these isoforms may interact with separate constituents of the cytoplasmic signaling apparatus, for example. Such variation in a highly conserved and presumably important functional portion of this protein, therefore, implies a potential regulation by alternative splicing of TRAF-3's role as a signaling molecule.

Evidence for the alternative use of exons has not been limited to the TRAF-3 coding sequence, but was initially revealed in the 5'-UTR, through variation among cDNA clones in exons 2, 3, and 4-13. The significance of this phenomenon (which is quite common, at least with respect to exon 3), is uncertain. It should be noted, however, that the IIIb form of TRAF-3 contains an open reading frame which precedes the initiating methionine at IIIb nt position 224. Interestingly, exon 2 is in frame with the rest of TRAF-3. Thus, while the inclusion of exon 3 leads to an early stop in translation, its removal allows for the production of a longer TRAF-3 species, exons 1,2,4-13.

It is not immediately clear how the described genomic structure of CRAF1 encodes p55, since in standard cell biology, a transcript including both exon 1 and 3 would

- 96 -

terminate translation in the region of cDNA of exon 3 that contains a stop codon. However, p55 could be encoded by the gene described herein, due to alternative promoters, alternative transcription initiation, alternative translation initiation or internal ribosomal re-entry.

Alternative promoters (e.g., c-abl gene (Bernards, A., et al. 1988. *Oncogene* 2:297)) could be present if an undetected promoter exists in the 1-2 intron (such a promoter may be a "TATA-less" promoter (Smale, S. T. and D. Baltimore. 1989. *Cell* 57:103) in this G-C rich region (e.g., Bloom syndrome gene (Ellis, N. A., et al. 1995. *Cell* 83:655) and if this transcript initiated after the AUG that encodes p70. Another possibility is that an alternative promoter exists in the 2-3 intron (encoding a cDNA encoded by exons 3-13 and encoding the p55 isoforms) (e.g., CD23 gene (Yokota, A., et al. 1988. *Cell* 55:611). Whether or not an alternative promoter exists, p55 encoding transcripts could arise by alternative transcription initiation sites (Hall, L. R., et al. 1988. *J. Immunol.* 141:2781), which possibility is favored by the lack of strong transcription initiation sites in the putative 5'-UT of exon 1, such as the TATA box and the CCAAT box (Bucher, P. and E. N. Trifonov. 1988. *J of Biomolecular Structure & Dynamics* 5:1231; Bucher, P. and E. N. Trifonov. 1986. *NAR* 14:10009) identified in **Figures 10A-10D**. (e.g. as in CD45 gene). Alternative translation initiation sites have also been shown to exist in certain cases and could lead to the generation of the p55 peptide species. Another possibility is that an internal ribosomal re-initiation sites might exist, in the cDNA and lead to p55 peptide species by cap-independent translation (Jang, S. K., et al. 1988. *J. Virol.* 62:2636; Molla, A., et al. 1992. *Nature* 356:255; Pelletier, J. and N. Sonenberg. 1988. *Nature* 334:320. It should be noted that one or more of

- 97 -

these strategies could be combined with alternative splicing to yield TRAF-3 isoforms that initiate translation in mRNA encoded by exon 10, (Mosialos, G., et al. 1995. Cell 80:389).

5

If this is the case than there may be two initiation sites for TRAF-3. A second, and unrelated possibility is that such alternative exons, like alternative polyadenylation at the 3' terminus of many mRNAs, may have some processing or regulatory significance, for example, by influencing message stability.

10

These various isoforms may play a role in modulating signaling mediated by TRAF-3. Each isoform, for example, may participate in a different signaling pathway, and may have a different level of expression or activity in a given cell. Some forms may act to inhibit others, or may be present only at critical moments in cellular development. The over-expression of these isoforms, therefore, in various cell lines may reveal important functional differences between them.

15

20

METHODS

25

Human metaphase spreads were prepared from fibroblast line. Ramos clone 2G6.4CN 3F10. Chromosome 14 telomere probe was purchased from Oncor.

30

A λ -gt11 Raji cDNA library (Stratagene) was screened. Probed with a 5' probe: NotI-SacI and with a 3' probe: C26. Clones were isolated by plating phage according to standard procedures on lawns of E. coli and plaque purified.

35

A human genomic λ -fix library (Stratagene) was screened. Clones were isolated by plating phage according to standard procedures on lawns of E. coli (P2932) and plaque purified.

- 98 -

A human genomic 3.5x arrayed P1 artificial chromosome library was used. Average insert size is 100 kb. This was prepared by partial Sau3AI digest and ligated into PCYPAC2. The library was screened with CRAF1 specific probes. Clones were isolated by transforming E. coli (DH10B), printing colonies (6144 colonies/filter) onto high density nylon membranes (SureBlot, Oncor) using the Biomek 2000 Automated Laboratory Workstation equipped with a high density replicating system (Beckman), and processing filters as described by Olsen et al. (BioTechniques 14:116-23 (1993)). Probes were labeled using random primers and filters were hybridized according to standard conditions (Genomics 21:525-37 (1994)).

The following probes were used to screen cDNA and genomic libraries as well as Southern blots: IIIb (IIIb 1-2467) a 2.4 kb cDNA which contains exon 2 as well as the entire TRAF-3 open reading frame. NotI-SmaI (IIIb nt 26-128), a 102 bp restriction fragment which contains the 5' half of exon 2. NotI-SacI, a probe which represents the first 447 bp of IIIb. CE (Ib nt 12-205), a 193 bp probe derived from cDNA clone Ib which represents a part of the TRAF-3 5'-UTR and contains exon E. HincII-HincII (IIIb 606-1003). zfl (2-1 nt 158-82) (tggtcaaactggctaccctgtccacc Seq I.D. No. _____) is a carboxy-terminal oligonucleotide fragment of exon 1. ff7gen-fr8gen is a 4.1 kb genomic fragment generated by genomic PCR using primers ff7gen and fr8gen (Table 3) which spans exons 5 and 6. pst1c-r2glx is a 5.8 kb genomic fragment generated by genomic PCR using primers pst1c and r2glx which spans exons 8 to 10.

λ phage genomic clones were sequenced on an automated DNA sequencer (ABI) using 5.0 μ g per sequencing reaction. P1 clones were sequenced manually by cycle sequencing (Gibco Cycle Sequencing kit) using 1.5 μ g

- 99 -

template DNA under the following conditions: DNA initial
denaturation 95 °C 3 min; denaturation 95 °C 30 sec,
annealing 64 °C 30 sec, extension 70 °C 60 sec X 20
cycles; denaturation 95 °C 30 sec, extension 70 °C 60
sec X 15 cycles.

Genomic PCR was carried out using the Expand long
template PCR kit (Boehringer-Mannheim) as per the
manufacturers directions. Human genomic DNA was
extracted from Ramos cells as follows: 50 x 10⁶ cells
pelleted 1x and washed 2x in PBS 5 min at 500 G,
resuspended in 0.5 ml digestion buffer (100 mM NaCl, 10
mM TrisCl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS, 0.1 mg/ml
proteinase K), incubated with gentle shaking at 50 °C 18
hrs, phenol-chloroform extracted 3x, and dialyzed
against TE 24 hrs (Current Protocols 2.2, Ausubel et al.
1992). The following PCR conditions were used: initial
denaturation 94 °C 4 min x 1 cycle; denaturation 94 °C
30 sec, annealing 62 °C 30 sec, extension 68 °C 15 min
x 10 cycles; denaturation 94 °C 30 sec, annealing 62 °C
30 sec, extension 68 °C 15 min with 20 sec additional
extension per cycle x 30 cycles; final extension 68 °C
7 min. Genomic PCR products were gel purified
(Qiaquick), subcloned (except 7-8, which was sequenced
directly) into pCR 2.0 or 2.1 (Invitrogen), and
sequenced on an automated DNA sequencer (ABI) at the
Columbia Sequencing Center, using standard conditions
(0.5 µg per sequencing reaction).

RT-PCR of the zinc-finger complex and the 5'-UTR regions
were performed as follows. HIGM patients A and C, and
C's unaffected mother CM were used. Patient B and an
unaffected mother of another HIGM patient, RS, were
provided. Total RNA was extracted (RNeasy kit: Qiagen)
from EBV-immortalized human B cell lines from all five
donors. RNA was oligo-dT primed and reverse transcribed
into cDNA (SuperScript II, Gibco BRL). cDNA was

- 100 -

amplified by PCR in the 5'-UTR using primers df7 (IIIb nt 133-150) agcagaacgctgcggacc (Seq. I.D. No. _____) and fr8 (IIIb nt 681-664) cagtcaggacgcacacat (Seq. I.D. No. _____), and in the zinc finger complex using primers 3b210f (IIIb nt 209-227) ctcctcttttcctaaaatgg (Seq. I.D. No. _____) and 3b1930r (IIIb nt 1949-1921) agctacttatcagggatcg (Seq. I.D. No. _____). All RT-PCR products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. RT-PCR products were gel purified and ligated into pCR2.0 and 2.1 (Invitrogen). Ligation products were cloned in E. coli and clones were examined for insert.

Further Comments on CRAF1 peptide and CRAF1 peptide isoforms

Despite wide tissue distribution CRAF1 peptide appears to have distinct and essential role in contact-help. CD40 is broadly expressed in many types of cells. CRAF1 peptide is ubiquitously expressed. Targeted gene disruption of CRAF1 nucleic acid results in defective contact-dependent helper function. Despite "collateral" signaling pathways, CRAF1 peptide is essential for T helper function. Analysis of CRAF1 nucleic acid mRNA revealed alternative mRNA splicing. A variety of CRAF1 nucleic acid mRNA species were isolated. A variety of splicing events alters the number of zinc-finger like domains. The three CRAF1 peptide isoforms are predicted by cDNA encoding loss of Zn-finger encoding exons. What is the evidence that longer CRAF1 nucleic acid mRNA species is important in regulating CD40 signaling? The IIIb type species (with an appended amino terminal peptide domain) is expressed by activated B cells and fibroblasts by RT-PCR. Hyper IgM patients with normal CD40-L preferentially express p70. Two unrelated HIGM patients expressed abnormal ratio of p70 and p60 mRNA. HIGM patients may overexpress p70 encoding mRNA. They may lack an in frame stop codon in exon 3.

- 101 -

Therapeutics

5 CRAF1 peptide and variants thereof and isoforms thereof
combined with carriers or therapeutically acceptable
agents for delivery may be used as therapeutic
compositions or therapeutic pharmaceuticals for
administration to subjects. Dominant negative CRAF1
peptide may be used in gene therapy as immunomodulator
or immunosuppressive therapeutics. Such peptides may be
co-administered with other therapeutic genes. Peptide
10 minmetics or small molecules are examples of agents
which may be used to mimic inhibitory CRAF1 peptides.
Screening would be facilitated by identification of p70
interacting species by systems such as the two-hybrid
yeast system.

15

Genomic sequencing has been done using the genomic
clones as described in Figures 7A-7B, 8 and 9A-9C.

Table 3. Genomic PCR and sequencing primers

	exons	forward oligonucleotide primer	oligonucleotide sequence (Seq. I.D. No. ____)	reverse primer
5	F1-F2 (4-5)	3b375f	ccgtggaggacaagtacaagtgtgagaag (____)	ff7genrev
	F2-F3 (5-6)	ff7gen	aaatgtacagcgtgtcaagagagcatcg (____)	f1genrev
	F3-F4 (6-7)	f1gen	gagagaaattcttggtctcttcagatctattgtcgg (____)	fr8gen
10	F4-F5 (7-8)	f1g1	gccattttgaagaacttccatgtgtgcgtcc (____)	pst1rge
	F5-F6 (8-9)	pst1c	gcagaaacacgaagacacccgactgtccctgc (____)	fx5rev
	F6-F7 (9-10)	fx5	tgtcagagtgtgtcaatgccccagcacctg (____)	r2g1x
15	F7-F8 (10-11)	exonf1	acaaaccagcagatcaaggcccacgag (____)	r2new
	F8-F9 (11-12)	bf8gen2	catacaaagtttgcacaatcagatatgtagc (____)	r2new18
	F9-F10 (12-13)	3b1225f	gagcttgacaaggagatccggcccttc (____)	3b1650r

Table 3 continued

	exons	revers IIIb nt primer	oligonucleotide sequence
5	F1-F2 (4-5)	ff7genrev	acgatgctctctttgacacgctgtacattttggac (Seq I.D. No. ____)
	F2-F3 (5-6)	f1genrev	ccgacaatagatctgaagagccagaattttctctc (Seq I.D. No. ____)
	F3-F4 (6-7)	fr8gen	tgcagtcaggacgcacacatggaag (Seq I.D. No. ____)
10	F4-F5 (7-8)	pst1rge	aggacaccaccacgcagggacagtcg (Seq I.D. No. ____)
	F5-F6 (8-9)	fx5rev	caggtgctgggggcattgacacactctg (Seq I.D. No. ____)
15	F6-F7 (9-10)	r2g1x	ggaaaccttcttttcgagcgagttgc (Seq I.D. No. ____)
	F7-F8 (10-11)	r2new	gctacatatctgattgtgcaaactttgtatg (Seq I.D. No. ____)
	F8-F9 (11-12)	r2new18	ccggaagggccggatctccttgtcaagctc (Seq I.D. No. ____)
20	F9-F10 (12-13)	3b1650r	caaggaagcagggcatcatattctccac (Seq I.D. No. ____)

Numbering is based on clone IIIb which is deposited in the
GenomeBank.

Table 4. Genomic λ phage and P1 clone sequencing primers

	name	oligonucleotide sequence	clone sequenced	primer type
5	ef1	ggaaaatgaggcccaaagaagtgatgccac (Seq I.D. No. ____)	P1 #33	forward exon
	er2b	caggacagcgatccttagaagagtaggg (Seq I.D. No. ____)	P1 #33	reverse exon
	acigenrev	ttagtctgcagcgccaggagag (Seq I.D. No. ____)	λ #6	reverse exon
	if207	ctttcccaaagctgtgtttgtttcc (Seq I.D. No. ____)	λ #6	forward intron
	if469	aatgctccagaaatctctgagtc (Seq I.D. No. ____)	λ #6	forward intron
10	if521	ttgccttgctccaaagtagcagcatg (Seq I.D. No. ____)	λ #6	forward intron
	if626	tagagattagaatctggtatttcag (Seq I.D. No. ____)	λ #6	forward intron
	if794	gagtgccataacttagaggacagcg (Seq I.D. No. ____)	P1 #167	forward intron
	if875	tatctgtgccctaataatgtttgaac (Seq I.D. No. ____)	P1 #167	forward intron
	intronf1	tagtgctgcttttagggctgtagttagcc (Seq I.D. No. ____)	λ #9	forward intron
15	if1043	aagctaacagaaggcctatattgtg (Seq I.D. No. ____)	λ #9	forward intron
	if1184	tgtatttgatggaaggtggtgcagc (Seq I.D. No. ____)	P1 #167	forward intron
	if1359	ctggtgcagctttgctttcctaacc (Seq I.D. No. ____)	P1 #167	forward intron

REFERENCES**References for Background**

1. S. Paulie et al., Cancer Immunol. Immunother. 20, 23(1985); E.A. Clark and J.A. Ledbetter, Proc. Natl. Acad. Sci. U.S.A. 83, 4494 (1986); I. Stamenkovic, E.A. Clark, B. Seed, EMBO J. 8, 1403 (1989).
2. S. Lederman et al., J. Exp. Med. 175, 1091 (1992); R. J. Armitage et al., Nature 357, 80(1992); D. Graf, U. Korthauer, H.W. Mages, G. Senger, R.A. Kroccek, Eur. J. Immunol. 22, 3191 (1992); J.F. Gauchat et al., FEBS Lett. 315, 259 (1993).
3. S. Lederman et al., J. Immunol. 149, 3817 (1992).
4. D. Hollenbaugh et al., EMBO J. 11, 4313 (1992).
5. T. Tsukata, J. Wu, T. Honjo, Nature 364, 645 (1993); N.J. Holder et al., Eur. J. Immunol. 23, 2368 (1993); S.L. Parry, J. Hasbold, M. Holman, G.G.B. Klaus J. Immunol. 152, 2821 (1994).
6. M.J. Yellin et al., J. Immunol. 153, 666 (1994).
7. R.C. Allen et al., Science 259, 990 (1993); U. Korthäuer et al., Nature 361, 539 (1993); J.P. DiSanto et al., ibid., p.541; A. Aruffo et al., Cell 72, 291 (1993); R.E. Callard et al., J. Immunol. 153, 3295 (1994); N. Ramesh et al., Int. Immunol. 5, 769 (1993).
8. J. Xu et al., Immunity 1, 423 (1994).
9. T. Kawabe et al., ibid., p. 167.
10. F. Schrieve et al., J. Exp. Med. 169, 2043 (1989); E.A. Clark, K.H. Grabstein, G.L. Shu, J. Immunol. 148, 3327 (1992).
11. D.N. Hart and J.L. McKenzie, J. Exp. Med. 168, 157 (1988).
12. M.R. Alderson et al., ibid. 178, 669 (1993).
13. A.H. Galy and H. Spits, J. Immunol. 149, 775 (1992).
14. F.H. Durie, T.M. Foy, S.R. Masters, J.D. Laman, R.J. Noelle, Immunol. Today 15, 406 (1994).

15. S. Inui et al., *Eur. J. Immunol.* 20, 1747 (1990).

References for Detailed Description and Example 1 of
Experimental Details

- 5 1. Lederman, S., et al. 1992. *J. Exp. Med.* 175:1091.
2. Armitage, R. J., et al. 1992. *Nature* 357:80.
3. Lederman, S., et al. 1992. *J. Immunol.* 149:3817.
4. Graf, D., et al. 1992. *Eur. J. Immunol.* 22:3191.
5. Hollenbaugh, D., et al. 1992. *EMBO J.* 11:4313.
10 6. Spriggs, M. K., et al. 1992. *J. Exp. Med.* 176:1543.
7. Lane, P., et al. 1992. *Eur. J. Immunol.* 22:2573.
8. Hermann, P., et al. 1993. *Eur. J. Immunol.* 23:961.
9. Gauchat, J. F., et al. 1993. *FEBS Lett.* 315:259.
10. Covey, L. R., et al. 1994. *Mol. Immunol.* 31:471.
15 11. Lederman, S., et al. 1994. *J. Immunol.* 152:2163.
12. Rosen, F. S., et al. 1961. *Pediatrics* 28:182.
13. Mayer, L., et al. 1986. *N. Engl. J. Med.* 314:409.
14. Notarangelo, L. D., et al. 1992. *Immunodef. Rev.*
3:101.
20 15. Fuleihan, R., et al. 1993. *Proc. Natl. Acad. Sci. U.
S. A.* 90:2170.
16. Korthauer, U., et al. 1993. *Nature* 361:539.
17. DiSanto, J. P., et al. 1993. *Nature* 361:541.
18. Allen, R. C., et al. 1993. *Science* 259:990.
25 19. Aruffo, A., et al. 1993. *Cell* 72:291.
20. Facchetti, F., et al. 1995. *J. Immunol.* 154:6624.
21. Ramesh, N., et al. 1993. *Int. Immunol.* 5:769.
22. Callard, R. E., et al. 1994. *J. Immunol.* 153:3295.
23. Xu, J., et al. 1994. *Immunity* 1:423.
30 24. Renshaw, B. R., et al. 1994. *Journal of Experimental
Medicine* 180:1889.
25. Kawabe, T., et al. 1994. *Immunity* 1:167.
26. Castigli, E., et al. 1994. *PNAS USA* 91:12135.
27. Noelle, R. J., et al. 1992. *Proc. Natl. Acad. Sci.
U. S. A.* 89:6550.
35 28. Van den Eertwegh, A. J. M., et al. 1993. *J. Exp.
Med.* 178:1555.

29. Foy, T. M., et al. 1993. *J. Exp. Med.* 178:1567.
31. Clark, E. A. and J. A. Ledbetter. 1994. *Nature* 367:425.
32. Noelle, R. J. and E. C. Snow. 1990. *Immunol. Today* 11:361.
- 5 33. Springer, T. A. 1990. *Nature* 346:425.
34. Lederman, S. and L. Chess. 1987. *Ann. Inst. Pasteur Immunol.* 138:158.
35. Lederman, S., et al. 1993. *Curr. Opin. Immunol.* 5:439.
- 10 36. Klaus, S. J., et al 1994. *J. Immunol.* 152:5643.
37. Valentine, M. A. and K. A. Licciardi. 1992. *Eur. J. Immunol.* 22:3141.
38. Liu, Y. J., et al 1989. *Nature* 342:929.
- 15 39. Liu, Y. J., et al. 1991. *Eur. J. Immunol.* 21:1905.
40. Tsubata, T., et al 1993. *Nature* 364:645.
41. Holder, M. J., et al. 1993. *Eur. J. Immunol.* 23:2368.
42. Casamayor-Palleja, C., et al. 1995. *J. Exp. Med.* 181:1293.
- 20 43. Stamenkovic, I., et al. 1989. *EMBO J.* 8:1403.
44. Smith, C. A., et al. 1994. *Cell* 76:959.
45. Browning, J. L., et al. 1993. *Cell* 72:847.
46. Suda, T., et al. 1993. *Cell* 75:1169.
- 25 47. Goodwin, R. G., et al. 1993. *Cell* 73:447.
48. Bowman, M. R., et al. 1994. *J. Immunol.* 152:1756.
49. Hintzen, R. Q., et al. 1994. *J. Immunol.* 152:1762.
50. Smith, C. A., et al. 1993. *Cell* 73:1349.
51. Goodwin, R. G., et al. 1993. *Eur. J. Immunol.* 23:2631.
- 30 52. Tartaglia, L. A. and D. V. Goeddel. 1992. *Immunol. Today* 13:151.
53. Trauth, B. C., et al. 1989. *Science* 245:301.
54. Itoh, N., et al 1991. *Cell* 66:233.
- 35 55. Camerini, D., et al. 1991. *J. Immunol.* 147:3165.
56. Pollok, K. E., et al. 1993. *J. Immunol.* 150:771.
57. Crowe, P. D., et al. 1994. *Science* 264:707.

- 108 -

58. Mallett, S., et al. 1990. *EMBO J.* 9:1063.
59. De Togni, P., et al. 1994. *Science* 264:703.
60. Jelinek, D. F. and P. E. Lipsky. 1987. *J. Immunol.* 139:2970.
- 5 61. Boussiotis, V. A., et al. 1994. *PNAS USA* 91:7007.
62. Aversa, G., et al. 1993. *J. Exp. Med.* 177:1575.
63. Macchia, D., et al. 1993. *Nature* 363:464.
64. Watanabe Fukunaga, R., et al. 1992. *Nature* 356:314.
65. Chu, J. L., et al. 1993. *J. Exp. Med.* 178:723.
- 10 66. Adachi, M., et al 1993. *Proc. Natl. Acad. Sci. U. S. A.* 90:1756.
67. Rieux-Laucat, F., et al. 1995. *Science* 268:1347.
68. Cohen, P. L. and R. A. Eisenberg. 1991. *Annu. Rev. Immunol.* 9P243-69.243.
- 15 69. Reap, E. A., et al. 1995. *J of Immun* 154:936.
70. Dhein, J., et al. 1995. *Nature* 373:438.
71. Brunner, T., et al. 1995. *Nature* 373:441.
72. Ju, S. T., et al. 1993 *Nature* 373:444.
73. Liu, Y., et al. 1992. *Immunol. Today* 13:17.
- 20 74. Conley, M. E., et al. 1994. *J. Clin. Invest.* 94:1404.
75. Cheng, G., et al. 1995. *Science* 267:1494.
76. Yellin, M. J., et al. 1991. *J. Immunol.* 147:3389.
77. Rothe, M., et al. 1994. *Cell* 78:681.
- 25 78. Grabstein, K. H., et al. 1993. *J. Immunol.* 150:tl)3141.
79. Lane, P., et al. 1993. *J. Exp. Med.* 177:1209.
80. Maliszewski, C. R., et al. 1993. *Eur. J. Immunol.* 23:1044.
- 30 81. Armitage, R. J., et al. 1993. *J. Immunol.* 150:3671.
82. Durandy, A., et al. 1993. *Eur. J. Immunol.* 23:2294.
83. Jung, S., et al. 1993. *Science* 259:984.
84. Gascan, H., et al. 1991. *J. Exp. Med.* 173:747.
85. Gauchat, J. F., et al. 1992. *J. Immunol.* 148:2291.
- 35 86. Schultz, C. L., et al. 1992. *J. Immunol.* 149:60.
87. Jumper, M. D., et al. 1994. *J. Immunol.* 152:438.
88. Yellin, M. J., et al. 1994. *J. Immunol.* 153:666.

89. Linsley, P. S., et al. 1992. *Science* 257:792.
90. Ranheim, E. A. and T. J. Kipps. 1993. *J. Exp. Med.* 177:925.
91. Fluckiger, A. C., et al. 1992. *Blood* 80:3173.
- 5 92. Yellin, M. J., et al. 1994. *J. Immunol.* 152:598.
93. Ktistakis, N. T., et al. 1990. *J. Cell Biol.* 111:1393.
95. Lagresle, C., et al. 1995. *Eur. J. Immunol.* 154:5746.
- 10 96. Inui, S., et al. 1990. *Eur. J. Immunol.* 20:1747.
97. Siegel, J. P. and H. S. Mostowski. 1990. *J. Immunol. Methods* 132:287.
98. Mosialos, G., et al. 1995. *Cell* 80:389. (Abstract)
99. Hu, H. M., et al. 1994. *J Biol Chem* 269:30069.
- 15 100. Hsu, H., et al. 1995. *Cell* 81:495.
101. Stanger, B. Z., et al. 1995. *Cell* 81:513.
102. Chinnaiyan, A. M., et al. 1995. *Cell* 81:505.
103. Clark, E. A., et al. 1989. *J. Immunol.* 143:3873.
104. Splawski, J. B. and P. E. Lipsky. 1994. *Research in Immunology* 145:226.
- 20 105. Banchereau, J. and F. Rousset. 1991. *Nature* 353:678.
106. Clark, E. A., et al. 1988. *Eur. J. Immunol.* 18:451.
107. Barrett, T. B., et al. 1991. *J. Immunol.* 146:1722.
- 25 108. Koopman, G., et al. 1994. *J. Immunol.* 152:3760.
109. Sumimoto, S., et al. 1994. *Journal of Immunology* 153:2488.
110. Henderson, S., et al. 1991. *Cell* 65:1107.
111. Wang, F., et al. 1990. *J. Virol.* 64:2309.
- 30 112. Zhang, Q., et al. 1994. *European Journal of Immunology* 24:1467.
113. Martin, J. M., et al. 1993. *Journal of Virology* 67:5269.
114. Tewari, M. and V. M. Dixit. 1995. *J. Biol. Chem.* 270:3255.
- 35 115. Tewari, M., et al. 1995. *Cell* 81:801.
116. Gulbins, E., et al. 1995. *Immunity* 2:341.

- 110 -

117. Wang, X., et al. 1994. *Cell* 77:53.118. Darnell, J. E., Jr., et al. 1994. *Science* 264:1415.References for Example 2:

- 5 1. Lederman, S., et al. 1992. *J. Exp. Med.* 175:1091.
2. Armitage, R. J., et al. 1992. *Nature* 357:80.
3. Lederman, S., et al. 1992. *J. Immunol.* 149:3817.
4. Graf, D., et al. 1992. *Eur. J. Immunol.* 22:3191.
5. Hollenbaugh, D., et al. 1992. *EMBO J.* 11:4313.
- 10 6. Spriggs, M. K., et al. 1992. *J. Exp. Med.* 176:1543.
7. Lane, P., et al. 1992. *Eur. J. Immunol.* 22:2573.
8. Hermann, P., et al. 1993. *Eur. J. Immunol.* 23:961.
9. Gauchat, J. F., et al. 1993. *FEBS Lett.* 315:259.
10. Covey, L. R., et al. 1994. *Mol. Immunol.* 31:471.
- 15 11. Rosen, F. S., et al. 1961. *Pediatrics* 28:182.
12. Mayer, L., et al. 1986. *N. Engl. J. Med.* 314:409.
13. Notarangelo, L. D., et al. 1992. *Immunodef. Rev.* 3:101.
14. Fuleihan, R., et al. 1993. *Proc. Natl. Acad. Sci. U. S. A.* 90:2170.
- 20 15. Korthauer, U., et al. 1993. *Nature* 361:539.
16. DiSanto, J. P., et al. 1993. *Nature* 361:541.
17. Allen, R. C., et al. 1993. *Science* 259:990.
18. Aruffo, A., M. et al. 1993. *Cell* 72:291.
- 25 19. Facchetti, F., C. et al. 1995. *J. Immunol.* 154:6624.
20. Ramesh, N., R. et al. 1993. *Int. Immunol.* 5:769.
21. Callard, R. E., et al. 1994. *J. Immunol.* 153:3295.
22. Kawabe, T., et al. 1994. *Immunity* 1:167.
23. Castigli, E., et al. 1994. *Proc Natl Acad Sci USA*
- 30 91:12135.
24. Xu, J., et al. 1994. *Immunity* 1:423.
25. Renshaw, B. R. et al. 1994. *J Exp Med* 180:1889.
26. Cheng, G., et al. 1995. *Science* 267:1494.
27. Mosialos, G., et al. 1995. *Cell* 80:389.
- 35 28. Hu, H. M., et al. 1994. *J Biol Chem* 269:30069.
29. Sato, T., S. Irie, and J. C. Reed. 1995. *FEBS Letters* 358:113.

- 111 -

30. Lederman, S., et al. 1994. *J. Immunol.* 152:2163.
31. Inui, S., et al. 1990. *Eur. J. Immunol.* 20:1747.
32. Valentine, M. A. and K. A. Licciardi. 1992. *Eur. J. Immunol.* 22:3141.
- 5 33. Liu, Y. J., et al. 1989. *Nature* 342:929.
34. Liu, Y. J., et al. 1991. *Eur. J. Immunol.* 21:1905.
35. Tsubata, T., et al. 1993. *Nature* 364:645.
36. Holder, M. J., et al. 1993. *Eur. J. Immunol.* 23:2368.
- 10 37. Conley, M. E., et al. 1994. *J. Clin. Invest.* 94:1404.
38. Rieux-Laucat, F., et al. 1995. *Science* 268:1347.
39. Durandy, A., et al. 1993. *Eur. J. Immunol.* 23:2294.
40. Ranheim, E. A. and T. J. Kipps. 1993. *J. Exp. Med.* 177:925.
- 15 41. Rothe, M., et al. 1994. *Cell* 78:681.
42. Wang, X., et al. 1994. *Cell* 77:53.
43. Darnell, J. E., et al. 1994. *Science* 264:1415.
44. Zhang, J., et al. 1994. *Oncogene* 9:1931.
- 20 45. Neri, A., et al. 1991. *Cell* 67:1075.
46. Chang, C. C., et al. 1994. *Oncogene* 9:923.
47. Cuomo, L., et al. 1993. *International Journal of Cancer* 53:1008.
48. Coney, L. R., et al. 1994. *International Journal of Cancer* 58:562.
- 25 49. Siegel, J. P. and H. S. Mostowski. 1990. *J. Immunol. Methods* 132:287.
50. Littman, D. R., et al. 1985. *Cell* 40:237.
51. Koopman, G., et al. 1994. *J. Immunol.* 152:3760.
- 30 52. Wyllie, A. H., et al. 1980. *Cytol.* 68:251.
53. Benhamou, L. E., et al. 1990. *Eur. J. Immunol.* 20:1405.

- 112 -

What is claimed is:

1. An isolated protein comprising a CRAF1-b domain
which comprises
5 GGARRGRRVREPGQLQPSRDFPAGGSRGGRRLFPAPRHGAARGAERCGPRRT
RPAPLSRPSGDGPPELLFPKM or a variant thereof capable
of inhibiting CD40-mediated cell activation.
2. The protein of claim 1, further comprising CRAF1-a
10 or a variant thereof adjacent to the carboxy-
terminus of the CRAF1-b domain.
3. The protein of claim 1, wherein the CRAF1-b domain
comprises at least 72 amino acids.
15
4. The protein of claim 1, wherein the CRAF1-b domain
comprises about 150 amino acids.
5. The protein of claim 1, wherein the variant
20 comprises a conservative amino acid substitution.
6. A CRAF1 peptide.
7. The peptide of claim 6, wherein the peptide
25 comprises an amino acid sequence encoded by exon X.
8. The peptide of claim 6, wherein the peptide
comprises an amino acid sequence encoded by exon Y.
9. A method of inhibiting activation by CD40 ligand of
30 cells expressing CD40 on the cell surface,
comprising providing the cells with the protein of
claim 1 or 6, the protein being present in an
amount effective to inhibit activation of the
35 cells.
10. The method of claim 9, wherein the cells are

- 113 -

provided with the protein by introducing into the cells a nucleic acid sequence encoding the protein under conditions such that the cells express an amount of the protein effective to inhibit activation of the cells.

5

10

15

20

25

30

35

11. The method of claim 10, wherein the nucleic acid sequence is operably linked to a transcriptional control sequence recognized by the cell.

12. The method of claim 10, wherein the nucleic acid sequence is a plasmid.

13. The method of claim 9, wherein the CD40-bearing cells are selected from the group consisting of B cells, fibroblasts, endothelial cells, epithelial cells, T cells, basophils, macrophages, Reed-Steinberg cells, dendritic cells, renal cells, and smooth muscle cells.

14. The method of claim 13, wherein the B cells are resting B cells, primed B cells, myeloma cells, lymphocytic leukemia B cells, or B lymphoma cells.

15. The method of claim 9, wherein the epithelial cells are keratinocytes.

16. The method of claim 9, wherein the fibroblasts are synovial membrane fibroblasts, dermal fibroblasts, pulmonary fibroblasts, or liver fibroblasts.

17. The method of claim 9, wherein the renal cells are selected from the group consisting of glomerular endothelial cells, mesangial cells, distal tubule cells, proximal tubule cells, parietal epithelial cells, visceral epithelial cells, cells of a Henle limb, and interstitial inflammatory cells.

- 114 -

18. The method of claim 17, wherein the parietal epithelial cells are crescent parietal epithelial cells.
- 5 19. The method of claim 9, wherein the smooth muscle cells are smooth muscle cells of the bladder, vascular smooth muscle cells, aortic smooth muscle cells, coronary smooth muscle cells, pulmonary smooth muscle cells, or gastrointestinal smooth muscle cells.
- 10 20. The method of claim 19, wherein the gastrointestinal smooth muscle cells are esophageal smooth muscle cells, stomachic smooth muscle cells, smooth muscle cells of the small intestine, or smooth muscle cells of the large intestine.
- 15 21. A method of providing a subject with an amount of the protein of claim 1 or 6 effective to inhibit activation by CD40 ligand of cells bearing CD40 on the cell surface in the subject, comprising:
introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding the protein of claim 1 or 6, under conditions such
20 that the cells express in the subject an activation inhibiting effective amount of the protein.
- 25 22. The method of claim 21, wherein the introducing of the nucleic acid into cells of the subject comprises:
30 a) treating cells of the subject ex vivo to insert the nucleic acid sequence into the cells; and
35 b) introducing the cells from step a) into the subject.

- 115 -

23. The method of claim 21, wherein the subject is a mammal.
24. The method of claim 23, wherein the mammalian
5 subject is a human.
25. The method of claim 21, wherein the CD40-bearing
cells are selected from the group consisting of B
cells, fibroblasts, endothelial cells, epithelial
10 cells, T cells, basophils, macrophages, Reed-
Steinberg cells, dendritic cells, renal cells, and
smooth muscle cells.
26. The method of claim 25, wherein the B cells are
15 resting B cells, primed B cells, myeloma cells,
lymphocytic leukemia B cells, or B lymphoma cells.
27. The method of claim 25, wherein the epithelial
cells are keratinocytes.
- 20 28. The method of claim 25, wherein the fibroblasts are
synovial membrane fibroblasts, dermal fibroblasts,
pulmonary fibroblasts, or liver fibroblasts.
- 25 29. The method of claim 25, wherein the renal cells are
selected from the group consisting of glomerular
endothelial cells, mesangial cells, distal tubule
cells, proximal tubule cells, parietal epithelial
cells, visceral epithelial cells, cells of a Henle
30 limb, and interstitial inflammatory cells.
30. The method of claim 29, wherein the parietal
epithelial cells are crescent parietal epithelial
cells.
- 35 31. The method of claim 25, wherein the smooth muscle
cells are smooth muscle cells of the bladder,

- 116 -

vascular smooth muscle cells, aortic smooth muscle cells, coronary smooth muscle cells, pulmonary smooth muscle cells, or gastrointestinal smooth muscle cells.

5

32. The method of claim 31, wherein the gastrointestinal smooth muscle cells are esophageal smooth muscle cells, stomachic smooth muscle cells, smooth muscle cells of the small intestine, or smooth muscle cells of the large intestine.

10

33. A method of treating a condition characterized by an aberrant or unwanted level of CD40-mediated intracellular signaling, in a subject, comprising providing the subject with a therapeutically effective amount of the protein of claim 1 or 6 capable of inhibiting CD40-mediated intracellular signaling in cells bearing CD40 on the cell surface.

15

20

34. The method of claim 33, wherein the protein is provided by introducing into CD40-bearing cells of the subject, a nucleic acid sequence encoding the protein under conditions such that the cells express the protein according to the method of claim 21.

25

35. The method of claim 33, wherein the condition is organ rejection in a subject receiving transplant organs, or an immune response in a subject receiving gene therapy.

30

36. The method of claim 35, wherein the transplant organ is a kidney, heart or liver.

35

37. The method of claim 35, wherein the condition is a CD40-dependent immune response.

- 117 -

38. The method of claim 37, wherein the CD40-dependent immune response is an autoimmune response in a subject suffering from an autoimmune disease.
- 5 39. The method of claim 38, wherein the autoimmune disease comprises rheumatoid arthritis, Myasthenia gravis, systemic lupus erythematosus, Graves' disease, idiopathic thrombocytopenia purpura, hemolytic anemia, diabetes mellitus, a drug-induced
10 autoimmune disease, psoriasis, or hyper IgE syndrome.
40. The method of claim 39, wherein the drug-induced autoimmune disease is drug-induced lupus.
- 15 41. The method of claim 39, wherein the immune response comprises induction of CD23, CD80 upregulation, rescue from CD95-mediated apoptosis, rescue from apoptosis in a subject undergoing chemotherapy
20 against a tumor, or autoimmune manifestations of an infectious disease.
42. The method of claim 41, wherein the autoimmune manifestations are derived from Reiter's syndrome, spondyloarthritis, Lyme disease, HIV infections,
25 syphilis or tuberculosis.
43. The method of claim 34, wherein the condition is an allergic response.
- 30 44. A method of claim 43, wherein the allergic response is hay fever or a penicillin allergy.
45. The method of claim 35, wherein the condition is dependent on CD40 ligand-induced activation of
35 fibroblast cells.

- 118 -

46. The method of claim 45, wherein the condition is selected from the group consisting of arthritis, scleroderma, and fibrosis.
- 5 47. The method of claim 46, wherein the arthritis is rheumatoid arthritis, non-rheumatoid inflammatory arthritis, arthritis associated with Lyme disease, or osteoarthritis.
- 10 48. The method of claim 46, wherein the fibrosis is pulmonary fibrosis, hypersensitivity pulmonary fibrosis, or a pneumoconiosis.
- 15 49. The method of claim 48, wherein the pulmonary fibrosis is pulmonary fibrosis secondary to adult respiratory distress syndrome, drug-induced pulmonary fibrosis, idiopathic pulmonary fibrosis, or hypersensitivity pneumonitis.
- 20 50. The method of claim 48, wherein the pneumoconiosis is asbestosis, siliconosis, or Farmer's lung.
- 25 51. The method of claim 43, wherein the fibrosis is a fibrotic disease of the liver or lung.
- 30 52. The method of claim 51, wherein the fibrotic disease of the lung is caused by rheumatoid arthritis or scleroderma.
- 35 53. The method of claim 51, wherein the fibrotic disease of the liver is selected from the group consisting of:
 Hepatitis-C;
 Hepatitis-B;
 cirrhosis;
 cirrhosis of the liver secondary to a toxic insult;

- 119 -

cirrhosis of the liver secondary to drugs;
cirrhosis of the liver secondary to a viral
infection; and
cirrhosis of the liver secondary to an
autoimmune disease.

5

54. The method of claim 53, wherein the toxic insult is
alcohol consumption.

10

55. The method of claim 53, wherein the viral infection
is Hepatitis B, Hepatitis C, or hepatitis non-B
non-C.

15

56. The method of claim 53, wherein the autoimmune
disease is primary biliary cirrhosis, or Lupoid
hepatitis.

20

57. The method of claim 34, wherein the condition is
dependent on CD40 ligand-induced activation of
endothelial cells.

25

58. The method of claim 57, wherein the condition is
selected from the group consisting of
atherosclerosis, reperfusion injury, allograft
rejection, organ rejection, and chronic
inflammatory autoimmune diseases.

30

59. The method of claim 58, wherein the atherosclerosis
is accelerated atherosclerosis associated with
organ transplantation.

35

60. The method of claim 58, wherein the chronic
inflammatory autoimmune disease is vasculitis,
rheumatoid arthritis, scleroderma, or multiple
sclerosis.

61. The method of claim 34, wherein the condition is

- 120 -

dependent on CD40 ligand-induced activation of epithelial cells.

5 62. The method of claim 61 wherein the epithelial cells are keratinocytes, and the condition is psoriasis.

63. The method of claim 34, wherein the condition is an inflammatory kidney disease.

10 64. The method of claim 63, wherein the inflammatory kidney disease is not initiated by autoantibody deposition in kidney.

15 65. The method of claim 63, wherein the kidney disease is selected from the group consisting of:
 membranous glomerulonephritis;
 minimal change disease/acute tubular necrosis;
 pauci-immune glomerulonephritis;
 focal segmental glomerulosclerosis;
20 interstitial nephritis;
 antitissue antibody-induced glomerular injury;
 circulating immune-complex disease;
 a glomerulopathy associated with a multisystem disease; and
25 drug-induced glomerular disease.

66. The method of claim 65, wherein the antitissue antibody-induced glomerular injury is anti-basement membrane antibody disease.

30 67. The method of claim 65, wherein the circulating immune-complex disease is selected from the group consisting of:

35 infective endocarditis;
 leprosy;
 syphilis;
 hepatitis B;

- 121 -

malaria; and
a disease associated with an endogenous antigen.

- 5 68. The method of claim 67, wherein the endogenous antigen is DNA, thyroglobulin, an autologous immunoglobulin, erythrocyte stroma, a renal tubule antigen, a tumor-specific antigen, or a tumor-associated antigen.
- 10 69. The method of claim 65 wherein the glomerulopathy associated with a multisystem disease is selected from the group consisting of:
- 15 diabetic nephropathy;
 systemic lupus erythematosus;
 Goodpasture's disease;
 Henoch-Schönlein purpura;
 polyarteritis;
 Wegener's granulomatosis;
- 20 cryoimmunoglobulinemia;
 multiple myeloma;
 Waldenström's macroglobulinemia; and
 amyloidosis.
- 25 70. The method of claim 65, wherein the pauci-immune glomerulonephritis is ANCA+ pauci-immune glomerulonephritis, or Wegener's granulomatosis.
- 30 71. The method of claim 65, wherein the interstitial nephritis is drug-induced interstitial nephritis.
72. The method of claim 63 wherein the kidney disease affects renal tubules.
- 35 73. The method of claim 72, wherein the kidney disease which affects renal tubules is selected from the group consisting of:

- 122 -

a kidney disease associated with a toxin;
a neoplasia;
hypersensitivity nephropathy;
Sjögren's syndrome; and
AIDS.

5

74. The method of claim 34, wherein the condition is a smooth muscle cell-dependent disease.
- 10 75. The method of claim 74, wherein the smooth muscle cell-dependent disease is a vascular disease.
76. The method of claim 75, wherein the vascular disease is atherosclerosis.
- 15 77. The method of claim 74, wherein the smooth muscle cell-dependent disease is a gastrointestinal disease.
- 20 78. The method of claim 77, wherein the gastrointestinal disease is selected from the group consisting of esophageal dysmotility, inflammatory bowel disease, and scleroderma.
- 25 79. The method of claim 74, wherein the smooth muscle cell-dependent disease is a bladder disease.
80. The method of claim 34, wherein the condition is associated with Epstein-Barr virus.
- 30 81. The method of claim 80, wherein the condition is selected from the group consisting of mononucleosis, B cell tumors, Burkitt's lymphoma, and nasopharyngeal carcinoma.
- 35 82. The method of claim 34, wherein the treatment does not increase susceptibility of the subject to

- 123 -

pneumocystis pneumonia, atypical infections, or tumors.

- 5 83. An isolated nucleic acid molecule encoding the protein of claim 1 or 6.
84. The nucleic acid molecule of claim 83, wherein the molecule is DNA.
- 10 85. A vector comprising the nucleic acid molecule of claim 83 operably linked to a transcriptional control sequence recognized by a host cell transformed with the vector.
- 15 86. The vector of claim 85, wherein the vector is a plasmid.

1/30

FIG. 1A

AAAGTCCTTTCTGTCTCCTCACCACCTGACTTGCAAACCTCCAGCGAAGGCCTCACCCCTCA
 1 -----+-----+-----+-----+-----+-----+ 60
 TTTCAGGAAAGACAGAGGAGTGGTGACTGAACGTTTGAGGTCGCTTCCGGAGTGCGGAGT

GCTGGCCAGAGCCTCCAGAGCCCTCCCTTCGCTTCCCACCTCGCCAGGCCAGCCAG
 61 -----+-----+-----+-----+-----+-----+ 120
 CGACCGGGTCTCGGAGGGTCTTCGGGAGGGAAGCGAAGGTGGAGCGGTCCGGGTCCGGTC

GGTGGGTGCTGCCTTGGTGGCCTAGGTGAATCACTGACACCACTGATGCCTTTGGGG
 121 -----+-----+-----+-----+-----+-----+ 180
 CCACCCAGACGGAACCAACCCGGATCCACTTAGTGACTGTGTGTTGACTACGGAAACCCC

Start of p70-i peptide (and p70 isoforms) - M P L G - 4

Start of putative peptide, p5 - M P L G - 4

Start of putative peptide, p15 - M P L G - 4

CAGAAGGCAGAGGCCTCGGAGAAATGCGAGGCCCCAGCGGCCTGGTGGAGGGTGCAGG
 181 -----+-----+-----+-----+-----+-----+ 240
 GTCTTCGGTCTCCGGAGCCCTCTTTACGGTCCGGGGTCCCGGACCACTCCACGTCC

p70-i Q K A E A S G E M R R P Q R P G G G C R - 24

p5 Q K A E A S G E M R R P Q R P G G G C R - 24

p15 Q K A E A S G E M R R P Q R P G G G C R - 24

· NSDOCID: <WO 9734473A1>

FIG. 1C

```

421  C C C C G C C A T G G G G C A G C C C G G G G A G C A A A A T G C T G C G G A C C G C G G G G A G G A C G C G C C G
      - - - - - + - - - - - + - - - - - + - - - - - + - - - - - +
      G G G G C G T A C C C C G T C G G G C C C C T C G T T T A C G A C G C C T G G C G C C C T C C T G C G C G G G C
      - - - - - + - - - - - + - - - - - + - - - - - + - - - - - +
p70-i P R H G A A R G A K C C G P R R R T R P - 104

p5

p15  P R H G A A R G A K C C G P R R R T R P - 104

481  G C G C C C C T G A G C C G G C G G G C G G C C G C A A G A T G A G G A A A T G A G G C C C A A G A
      - - - - - + - - - - - + - - - - - + - - - - - + - - - - - +
      C G G G G G A C T C G G C C G G C T C G C G G T G C C T G G G T T C T A C T C C T T T T A C T C C G G G T T C T
      - - - - - + - - - - - + - - - - - + - - - - - + - - - - - +
p70-i A P L S R P S G D G P Q - 116
      2**3

p5      M R K M R P K E - 55

p15    A P L S R P S G D G P Q D E E N E A Q R - 124

541  A G T G A T G C C A C T T G G T T A A G G T C C C A G A G C A G G T C A G A A T C A G A C C T A G G A T C A G A A C C
      - - - - - + - - - - - + - - - - - + - - - - - + - - - - - +
      T C A C T A C G G T G A A C C A A T T C C A G G T C T C G T C C A G T C T T A G T C T G G A T C C T A G T C T T T G G
      - - - - - + - - - - - + - - - - - + - - - - - + - - - - - +
p70-i

p5      - V M P L G * stop - 60

p15     - * stop

```


4/30

FIG. 1D

TGGCTCCTGGCTCCTGGCTCCCTACTCTTCTAAGGATCGCTTGTCTCTGACAGAAGAAGAAC
 601 -----+-----+-----+-----+-----+ 660
 ACCGAGGACCGAGGACCGAGGGATGAGAAGATTCTCTAGCGAACAGGACTGTCTTCTCTTGG
 3**4
 E L - 118
 p70-i -

TCCTCTTTCCATAAATGGAGTCGAGTAAAGATGGACTCTCCTGGCGGCTGCAGACTA
 661 -----+-----+-----+-----+ 720
 AGGAGAAAGGATTTTACCTCAGCTCATTTTCTACCTGAGAGGACCGCGCGACTGTGAT

Start of p55-i M E S S K K M D S P G A L Q T N - 16
 p70-i L F P K M E S S K K M D S P G A L Q T N - 138

ACCGCGCGCTAAAGCTGCACACTGACCGTAGTGTGGGACGCCAGTTTGTCCCTGAAC
 721 -----+-----+-----+-----+ 780
 TGGCGGGCGATTTCGACGTGTGACTGGCATCACGACCCCTGCGGTCAAAAACAGGGACTTG

p55-i - P P L K L H T D R S A G T P V F V P E Q - 36
 p70-i - P P L K L H T D R S A G T P V F V P E Q - 158

AAGGAGGTTACAAGGAAAAGTTTGTGAAGACCGTGGAGGACAAGTACAAGTGTGAGAAGT
 781 -----+-----+-----+-----+ 840
 TTCCTCCAATGTTCTCTTTTCAAACACTTCTGGCACCTCCTGTTCATGTTTCACACTCTCA

p55-i - G G Y K E K F V K T V E D K Y K C E K C - 56
 p70-i - G G Y K E K F V K T V E D K Y K C E K C - 178

5/30

FIG. 1E

841 -----+-----+-----+-----+-----+-----+-----+ 900
 GCCACCTGGTGTGTGCAGCCCGAAGCAGACCGAGTGTGGCACCCTTCTGCGAGAGCT
 CCGTGGACCCAGACACGTCGGGCTTCGTCTGGCTCACACCCGTGGCGAAGACGCTCTCGA

 p55-i - H L V L C S P K Q T E C G H R F C E S C - 76
 p70-i - H L V L C S P K Q T E C G H R F C E S C - 198

 901 -----+-----+-----+-----+-----+-----+ 960
 GCATGGCGGCCCTGTGAGCTCTTCAAGTCCAAATGTACAGCGTGTCAAGAGAGCATCG
 CGTACCGCGGGACGACTCGAGAAGTTCAGGTTTACATGTCCACAGTTCTCTCGTAGC
 4**5
 p55-i M A A L L S S S P K C T A C Q E S I V - 96
 p70-i - M A A L L S S S P K C T A C Q E S I V - 218

 961 -----+-----+-----+-----+-----+-----+ 1020
 TTAAAGATAAGGTGTTTAAGGATAATTGCTGCAAGAGAGAAATTCTGGCTCTTCAGATCT
 AATTCTATTCCACAAATTCCTATTAAACGAGTTCTCTCTTTAAGACCGAGAAGTCTAGA
 5**6
 p55-i - K D K V F K D N C C K R E I L A L Q I Y - 116
 p70-i - K D K V F K D N C C K R E I L A L Q I Y - 238

6/30

FIG. 1F

```

ATTGTCGGAAATGAAAGCAGAGGTTGTGCAGAGCAGTTAACGCTGGGACATCTGCTGGTGC
1021 -----+-----+-----+-----+-----+-----+ 1080
TACAGCCTTACTTTCGCTCTCCAACACAGTCTCGTCAATTGCGACCCCTGTAGACGACCACG
6**7
p55-i - C R N E S R G C A E Q L T L G H L L V H - 136
p70-i - C R N E S R G C A E Q L T L G H L L V H - 258

ATTTAAAAATGATTGCCATTTTGAAGAACTTCCATGTGTGCGTCTGACTGCAAAAGAA
1081 -----+-----+-----+-----+-----+ 1140
TAAATTTTACTAACGGTAAAACTTCTTGAAGGTACACACGCAGGACTGACGTTTCTTT

p55-i - L K N D C H F E E L P C V R P D C K E K - 156
p70-i - L K N D C H F E E L P C V R P D C K E K - 278

AGGTCCTTGAGGAAAGACCTGCGAGACCACGTGGAGAGGCGTGTAAATACCGGAAGCCA
1141 -----+-----+-----+-----+-----+ 1200
TCCAGAACTCCTTCTGGACGCTCTGGTGACCTCTCCGCACATTATGGCCCTTCGGT

p55-i - V L R K D L R D H V E K A C K Y R E A T - 176
p70-i - V L R K D L R D H V E K A C K Y R E A T - 298

```

FIG. 1G

1201	CATGCAGCCACTGCAAGAGTCAGGTTCCGATGATCGCGCTGCAGAAACACGAAGACACCG	1260
	GTACGTCGGTGACGTTCTCAGTCCCAAGGCTACTAGCGGACGCTTTGTGCTTCTGTGGC	
	7**8	
p55-i -	C S H C K S Q V P M I A L Q K H E D T D - 196	
p70-i -	C S H C K S Q V P M I A L Q K H E D T D - 318	
p55del9 -	C S H C K S Q V P M I A L Q K H E D T D - 196	
p70del9 -	C S H C K S Q V P M I A L Q K H E D T D - 318	
p55del9,10-C	S H C K S Q V P M I A L Q K H E D T D - 196	
p70del9,10-C	S H C K S Q V P M I A L Q K H E D T D - 318	
p55del-8,9-C	S H C K S Q V P M I A L Q	- 190
p70del-8,9-C	S H C K S Q V P M I A L Q	- 312

	ACTGTCCCTGGTGGTGCCTCACAAGTGACGCGTCAGACTCTCCTGAGGA	1261	-----+-----+-----+-----+-----+-----+ 1320
	TGACAGGGACGCCACCACACAGGACGGGAGTGTTCAAGTCGACGGTCTGAGAGGACTCCT		
p55-i -	C P C V V V S C P H K C S V Q T L L R S - 216		
p70-i -	C P C V V V S C P H K C S V Q T L L R S - 338		
p55del9-	C P C V V V S C P H K C S V Q T L L R S - 216		
p70del9-	C P C V V V S C P H K C S V Q T L L R S - 338		
p55del9,10-C	P C V V V S C P H K C S V Q T L L R S - 216		
p70del9,10-C	P C V V V S C P H K C S V Q T L L R S - 338		
p55del8,9-			
p70del8,9-			

9/30

FIG. 11

1321	GGGAGTTGAGTGCACACTTGTTCAGAGTGTGTCAATGCCCCCAGCACCTGTAGTTTAAAGC	1380
	CGCTCAACTCACGTGTGAACAGTCTCACACAGTTACGGGGGTCTGGGACATCAAAAATTCG	
	8**9	
p55-i -	E L S A H L S E C V N A P S T C S F K R - 236	
p70-i -	E L S A H L S E C V N A P S T C S F K R - 358	
p55del9-	E	- 217
p70del9-	E	- 339
p55del9,10-E		- 217
p70del9,10-E		- 339
p55del8,9-		
p70del8,9-		

10/30

FIG. 1J

	GCTATGGCTGCGTTTTCAGGGGACAAACCAGCAGATCAAGGCCACGAGGCCAGCTCCG	1440
1381	CGATACCGACGCAAAAGTCCCCTGTTGGTCGCTAGTCCGGGTGCTCCGGTCGAGGC	
	9**10	
p55-i -	Y G C V F Q G T N Q Q I K A H E A S S A - 256	
p70-i -	Y G C V F Q G T N Q Q I K A H E A S S A - 378	
p55del9-	G T N Q Q I K A H E A S S A - 231	
p70del9-	G T N Q Q I K A H E A S S A - 353	
p55del9,10-		
p70del9,10-		
p55del18,9-	G T N Q Q I K A H E A S S A - 204	
p70del18,9-	G T N Q Q I K A H E A S S A - 326	

11/30

FIG. 1K

```
CCGTGCAGCACGTC AACCTGCTGAAGGAGTGGAGCAACTCGCTCGAAAAGAAGTTTCCT
1441 -----+-----+ 1500
GGCACGTCGTGCAGTTGGACGACTTCCTCACCTCGTTGAGCGAGCTTTTCTTCCAAAGGA
10**11
p55-i - V Q H V N L L K E W S N S L E K K V S L - 276
p70-i - V Q H V N L L K E W S N S L E K K V S L - 398
p55del9- V Q H V N L L K E W S N S L E K K V S L - 251
p70del9- V Q H V N L L K E W S N S L E K K V S L - 373
p55del9,10- V S L - 220
p70del9,10- V S L - 342
p55del18,9- V Q H V N L L K E W S N S L E K K V S L - 224
p70del18,9- V Q H V N L L K E W S N S L E K K V S L - 346

TGTTCAGAAATGAAAGTGTAGAAAAAACAAGAGCATACAAGTTGCACAATCAGATAT
1501 -----+-----+ 1560
ACAACGTCTTACTTTCACATCTTTTGTCTCGTATGTTTCAACGGTGTAGTCTATA
p55-i - L Q N E S V E K N K S I Q S L H N Q I C - 296*
p70-i - L Q N E S V E K N K S I Q S L H N Q I C - 418*
```


12/30

FIG. 1L

GTAGCTTTGAAATTGAAATTGAGAGACAAAGGAAATGCTTCGAAATAATGAATCCAAAA
 1561 -----+-----+-----+-----+-----+ 1620
 CATCGAAACTTTAACTTTAACTCTCTGTCTTTCCTTTACGAAGCTTTATTACTTAGGTTT

 p55-i - S F E I E I E R Q K E M L R N N E S K I - 316*
 p70-i - S F E I E I E R Q K E M L R N N E S K I - 438*

 TCCTTCATTACAGCGAGTGATCGACAGCCCAAGCAGAGAACTGAAGGAGCTTGACAAGG
 1621 -----+-----+-----+-----+-----+ 1680
 AGGAAGTAAATGTCGCTCACTAGCTGTGCGTTCGTCTCTTTGACTTCCTCGAACTGTTCC
 11**12
 p55-i - L H L Q R V I D S Q A E K L K E L D K E - 336*
 p70-i - L H L Q R V I D S Q A E K L K E L D K E - 458*

 AGATCCGGCCCTTCCGGCAGAACTGGGAGGAAGCAGACAGCATGAAGAGCAGCGTGGAGT
 1681 -----+-----+-----+-----+-----+ 1740
 TCTAGGCCGGGAAGGCGCTCTTGACCCCTCCTTCGTCTGCTGCTACTTCTCGTCGCACCTCA

 p55-i - I R P F R Q N W E E A D S M K S S V E S - 356*
 p70-i - I R P F R Q N W E E A D S M K S S V E S - 478*

 CCTTCCAGAACCGGTGACCGAGCTGGAGAGCGTGACAAAGAGTGCGGGGCAAGTGGCTC
 1741 -----+-----+-----+-----+-----+ 1800
 GGGAGGTCTTGGCGCACTGGCTCGACCTCTCGCACCTGTTCTCACGCCCGTTCACCCGAG

 p55-i - L Q N R V T E L E S V D K S A G Q V A R - 376*
 p70-i - L Q N R V T E L E S V D K S A G Q V A R - 498*

13/30

FIG. 1M

1801 GGAACACAGGCCTGCTGGAGTCCAGCTGA9CCGGCATGACCAGATGCTGAGTGTGCACG
 -----+-----+-----+-----+-----+ 1860
 CCTTGTGTCCGGACGACCTCAGGGTCGACTcGGCCGTACTGGTCTACGACTCACACGTGC
 12*13
 p55-i - N T G L L E S Q L S R H D Q M L S V H D - 396*
 p70-i - N T G L L E S Q L S R H D Q M L S V H D - 518*

1861 ACATCCGCCTAGCCGACATGGACCTGCGCTTCCAGGTCTTGAGACCGCCAGCTACAATG
 -----+-----+-----+-----+-----+ 1920
 TGTAGCGGATCGGCTGTACCTGGACGGGAAGGTCAGGACCTCTGGCGGTCTGATGTTAC
 p55-i - I R L A D M D L R F Q V L E T A S Y N G - 416*
 p70-i - I R L A D M D L R F Q V L E T A S Y N G - 538*

1921 GAGTGCTCATCTGGAAGATTGCGGACTACAAGCGGGGAAGCAGGAGGCCGTCATGGGGA
 -----+-----+-----+-----+-----+ 1980
 CTCACGAGTAGACCTTCTAAGCGCTGATGTTCCGCCCTTCGTCCTCCGGCAGTACCCCT
 p55-i - V L I W K I R D Y K R R K Q E A V M G K - 436*
 p70-i - V L I W K I R D Y K R R K Q E A V M G K - 558*

1981 AGACCCGTGCCCTTTACAGCCAGCCTTTCTACACTGGTTACTTTGGTTATAAGATGTGTG
 -----+-----+-----+-----+-----+ 2040
 TCTGGGACAGGGAATGTCCGGTCGGAAAGATGTGACCAATGAAACCAATATTCTACACAC
 p55-i - T L S L Y S Q P F Y T G Y F G Y K M C A - 456*
 p70-i - T L S L Y S Q P F Y T G Y F G Y K M C A - 578*

14/30

FIG. 1N

2041 CCAGGGTCTACCTGAACGGGGACGGGATGGGGAAGGGGACGCCACTTGTGCTGTTTTTTG
 -----+-----+-----+-----+-----+ 2100
 GGTCCCAGATGGACTTGCCCTGCCCTACCCCTTCCCTGCGTGAACAGCGACAAAAAC

 p55-i - R V Y L N G D G M G K G T H L S L F F V - 476*

 p70-i - R V Y L N G D G M G K G T H L S L F F V - 598*

 2101 TCATCATGCGTGGAGAATATGATGCCCTGCTTCCTTGGCCGTTAAGCAGAAAGTGACAC
 -----+-----+-----+-----+-----+ 2160
 AGTAGTACGCACCTCTTATACTACGGGACGAAGAACCGGCAATTCGTCTTCACCTGTG

 p55-i - I M R G E Y D A L L P W P F K Q K V T L - 496*

 p70-i - I M R G E Y D A L L P W P F K Q K V T L - 618*

 2161 TCATGCTGATGGATCAGGGGTCTCTCGACGTGCTTTGGGAGATGCATTCAAGCCCGACC
 -----+-----+-----+-----+-----+ 2220
 AGTACGACTACCTAGTCCCCAGGAGAGCTGCAGTAAACCCCTCTACGTAAGTTCGGGGCTGG

 p55-i - M L M D Q G S S R R H L G D A F K P D P - 516*

 p70-i - M L M D Q G S S R R H L G D A F K P D P - 638*

 2221 CCAACAGCAGCAGCTCAAGAAGCCCACTGGAGAGATGAATATCGCCTCTGGCTGCCCCAG
 -----+-----+-----+-----+-----+ 2280
 GGTGTCGTCGTCGAAGTTCTTCGGGTGACCTCTCTACTATAGCGGAGACCGACGGGTC

 p55-i - N S S S F K K P T G E M N I A S G C P V - 536*

 p70-i - N S S S F K K P T G E M N I A S G C P V - 658*

15/30

FIG. 10

2281 TCTTTGTGCCCCAAACTGTTCTAGAAAATGGACATATATTAAGATGATACAAATTTTA 2340
 AGAAACACCGGGTTTGACAAGATCTTTACCCCTGTATATAATTTCTACTATGTAAAAAT
 p55-i - F V A Q T V L E N G T Y I K D D T I F I - 556*
 p70-i - F V A Q T V L E N G T Y I K D D T I F I - 678*

2341 TTAAAGTCATAGTGATACCTCGGATCTGCCGATCCCTGATAAGTAGTGGGAGGTGG 2400
 AATTTCAGTATCACCTATGAAGCCTAGACGGGCTAGGGACTATTCATCGACCCCTCCACC
 p55-i - K V I V D T S D L P D * * -568*
 p70-i - K V I V D T S D L P D P * * -690*

2401 ATTTAGCAGAAGGCAACTCCTCTGGGGGATTGAACCGGTCTGTCTTCACTGAGGTCCTC 2460
 TAAATCGTCTTCCGTTGAGGAGACCCCTAAACTTGGCCAGACAGAGTGACTCCAGGAG

2461 GCGCTCAGAAAAGGACCTTGTGAGACGGAGGAAGCGGAGCGGACGCGTGCCGGCG 2520
 CGCGAGTCTTTCCCTGGAACACTCTGCCTCCTTCGCCGTCTTCCGCCCTGCCACGGCCGC

2521 GGAGGAGCCACGGTGAGCACACCTGACACGTTTATAATAGACTAGCCACACTTCACATC 2580
 CCTCCTCGGTGCGCACTCGTGTGGACTGTGCAAAATATATCTGATCGGTGTGAAGTGAG

16/30

FIG. 1P

```
2581  TGAAGAATTATTATCCTTCAACAAGATAAATATTGCTGTCAGAGAAGGTTTTCATTTTC
-----+-----+-----+-----+-----+-----+-----+
2640  ACTTCTTAATAAATAGGAAGTTGTTCTATTATAACGACAGTCTCTTCCAAAAGTAAAG
-----+-----+-----+-----+-----+-----+-----+

2641  ATTTTAAAGATCTAGTTAAATTAAGGTGGAAACATATATGCTAAACaAAAGAAACATGA
-----+-----+-----+-----+-----+-----+-----+
2700  TAAAAATTCTAGATCAATTAATCCACCCTTTTGATATACGATTGtTTCTTTGTACT
-----+-----+-----+-----+-----+-----+-----+

2701  TTTTCTTCCTTAAACTTGAACACCaaAAAAACACACACACACACGTTGGGATAGC
-----+-----+-----+-----+-----+-----+-----+
2760  AAAAAGAAGGAATTGAACCTTGTGGtTTTTTTGTGTGTGTGTGTGCACCCCTATCG
-----+-----+-----+-----+-----+-----+-----+

2761  TGGACATGTCAGCATGTTAAGTAAAGGAGAATTTATGAATAGTAATGCAATTCTGATA
-----+-----+-----+-----+-----+-----+-----+
2820  ACCTGTACAGTCGTACAATTTCATTTTCCTCTTAAATACTTTATCATTACGTTAAGACTAT
-----+-----+-----+-----+-----+-----+-----+

2821  TCCTCTTCTAAAATTCAAGAGTGCAATTTTGTTTTCAATACAGTATATTGTCTATTTT
-----+-----+-----+-----+-----+-----+-----+
2880  AGAAGAAAGATTTTAAGTTCTCACGTTAAACAAAGTTTATGTCATATAACAGATAAAA
-----+-----+-----+-----+-----+-----+-----+

2881  AAGCCCTCCAAAAAATAAATAATTCGGCCGGAATTC
-----+-----+-----+-----+-----+-----+-----+
2918  TTCCGGAGGTTTTTTTTTTTAAAGCCCGGCCTTAAG
-----+-----+-----+-----+-----+-----+-----+
```

Note: * on aa number, indicates that numbering is for p70 or p55 without internal deletions.

xx, indicates exon boundaries.

17/30

FIG. 2A

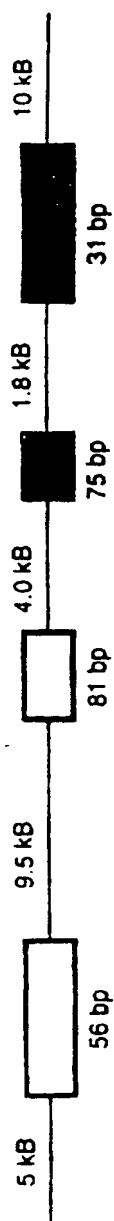
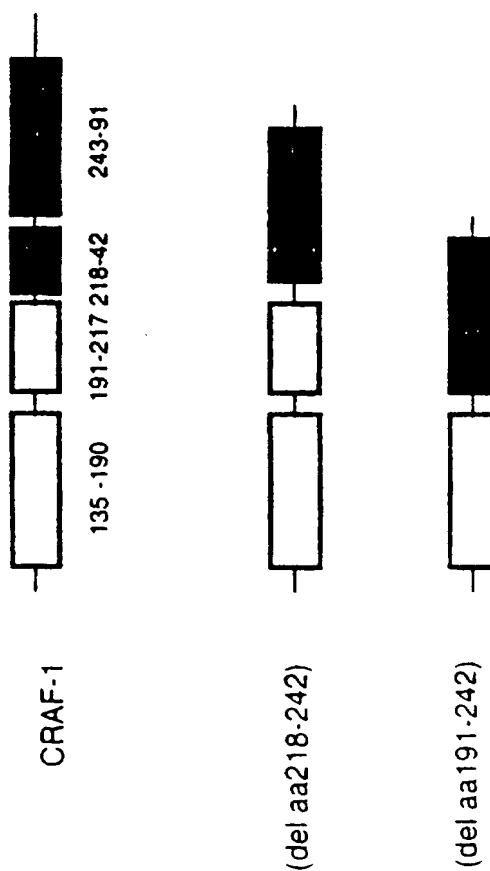


FIG. 2B



CRAF-1

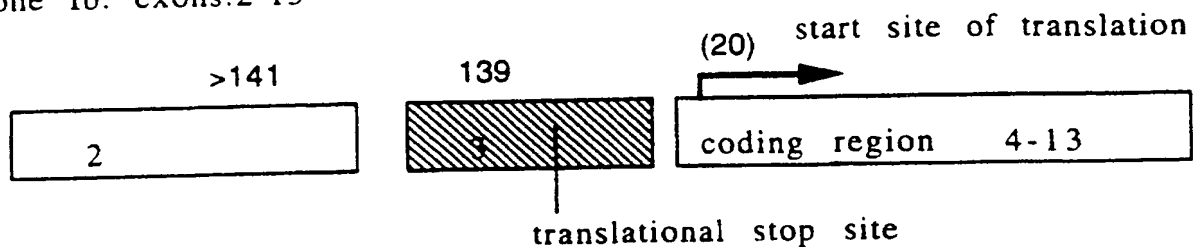
(del aa 218-242)

(del aa 191-242)

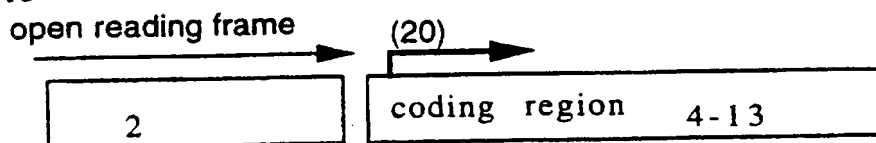
18/30

FIG. 3

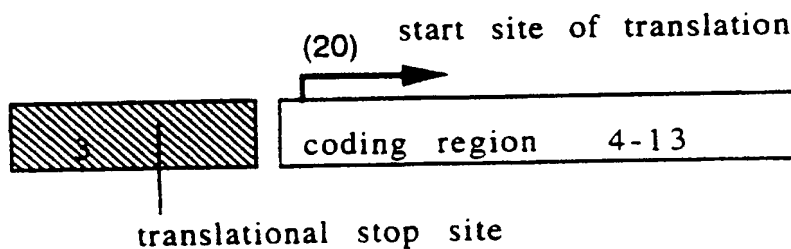
clone Ib: exons:2-13



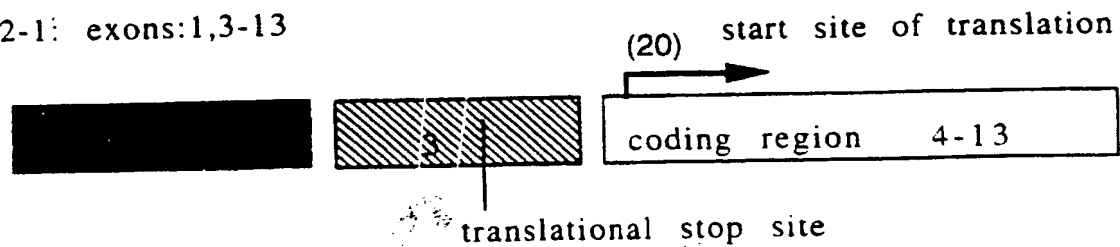
clone IIIb: exons:2,4-13



clone 5-2: exons:3-13



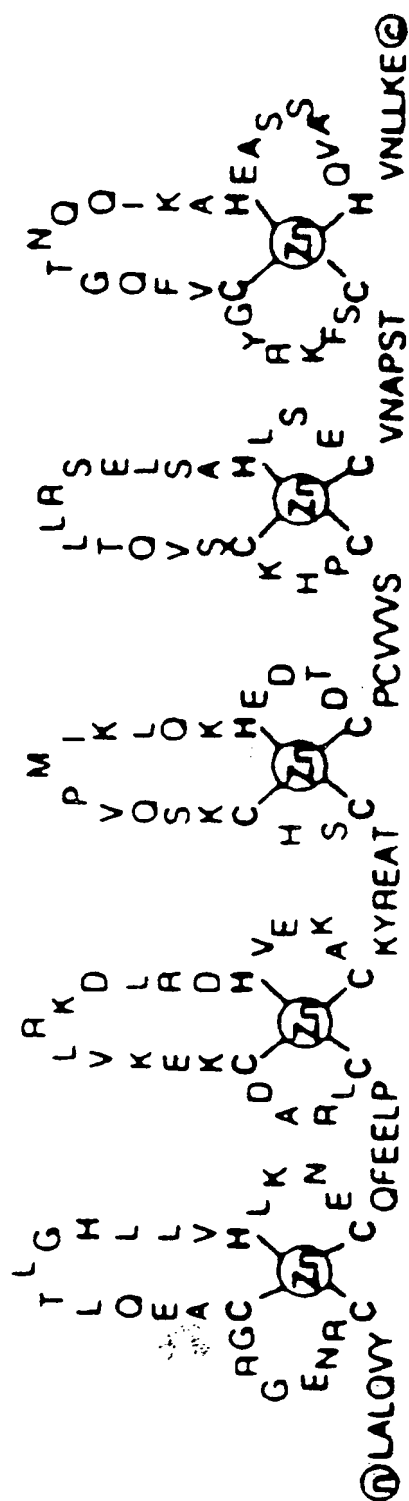
clone 2-1: exons:1,3-13



(Note that cDNA containing exons:1,2,4-13 have not been isolated)

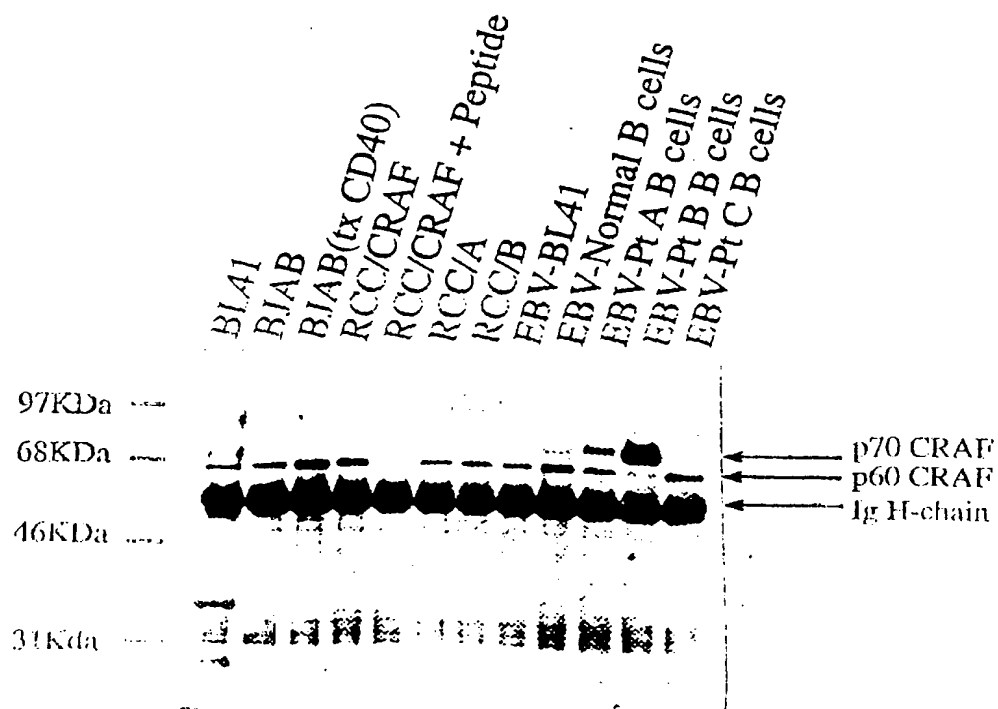
19/30

FIG. 4



20/30

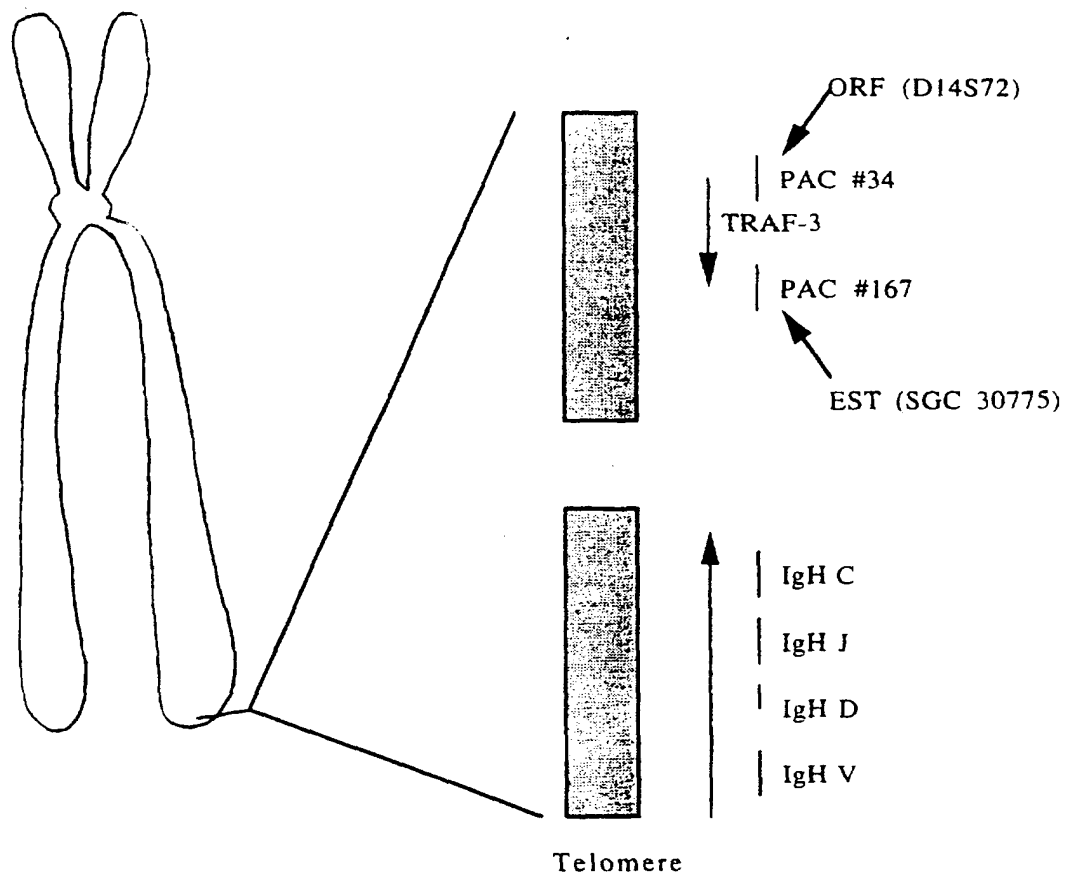
FIG. 5



SUBSTITUTE SHEET (RULE 26)

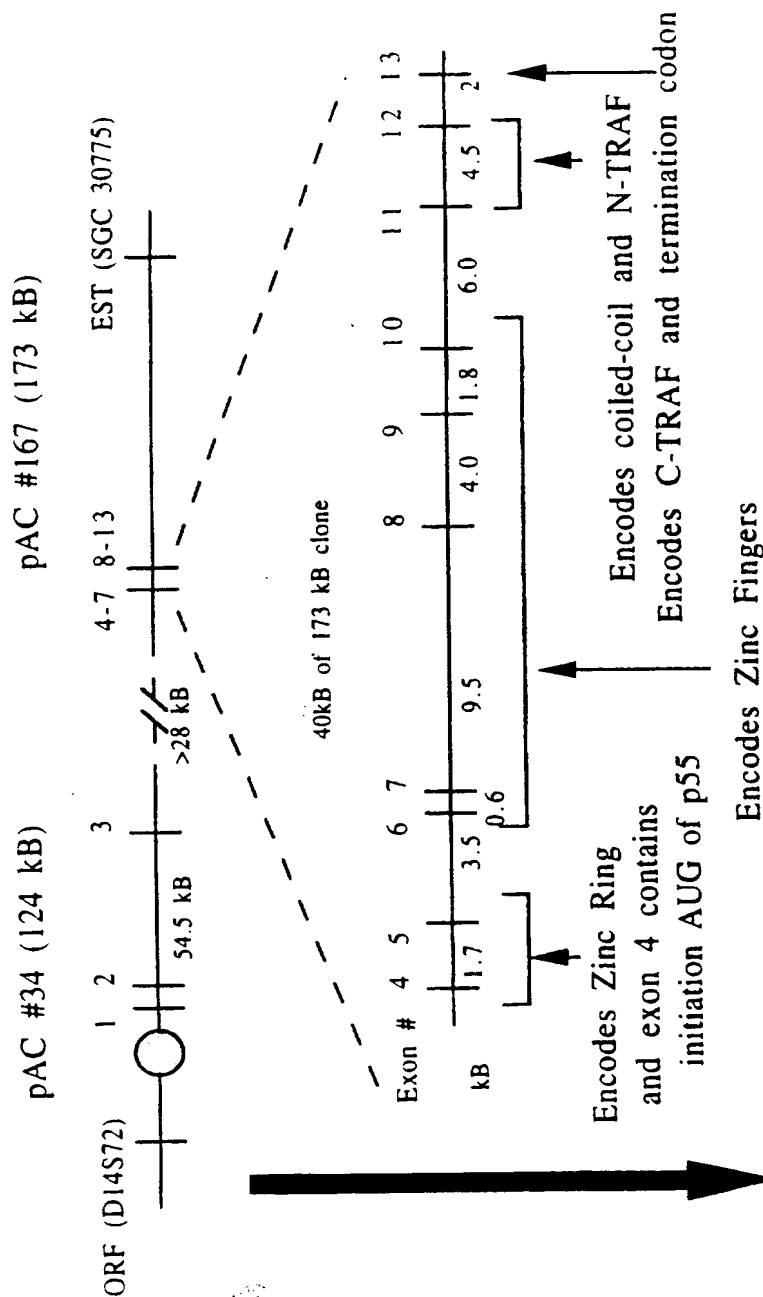
21/30

FIG. 6



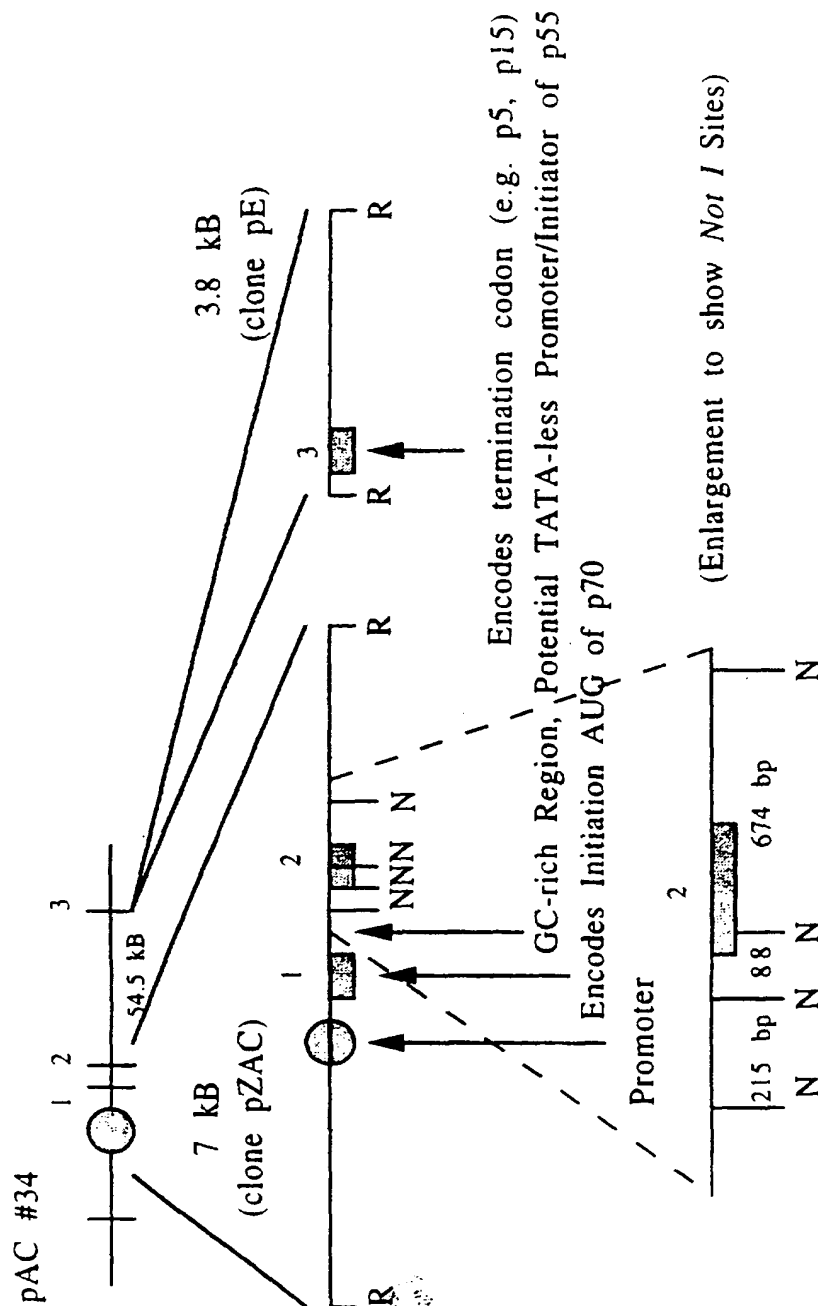
22/30

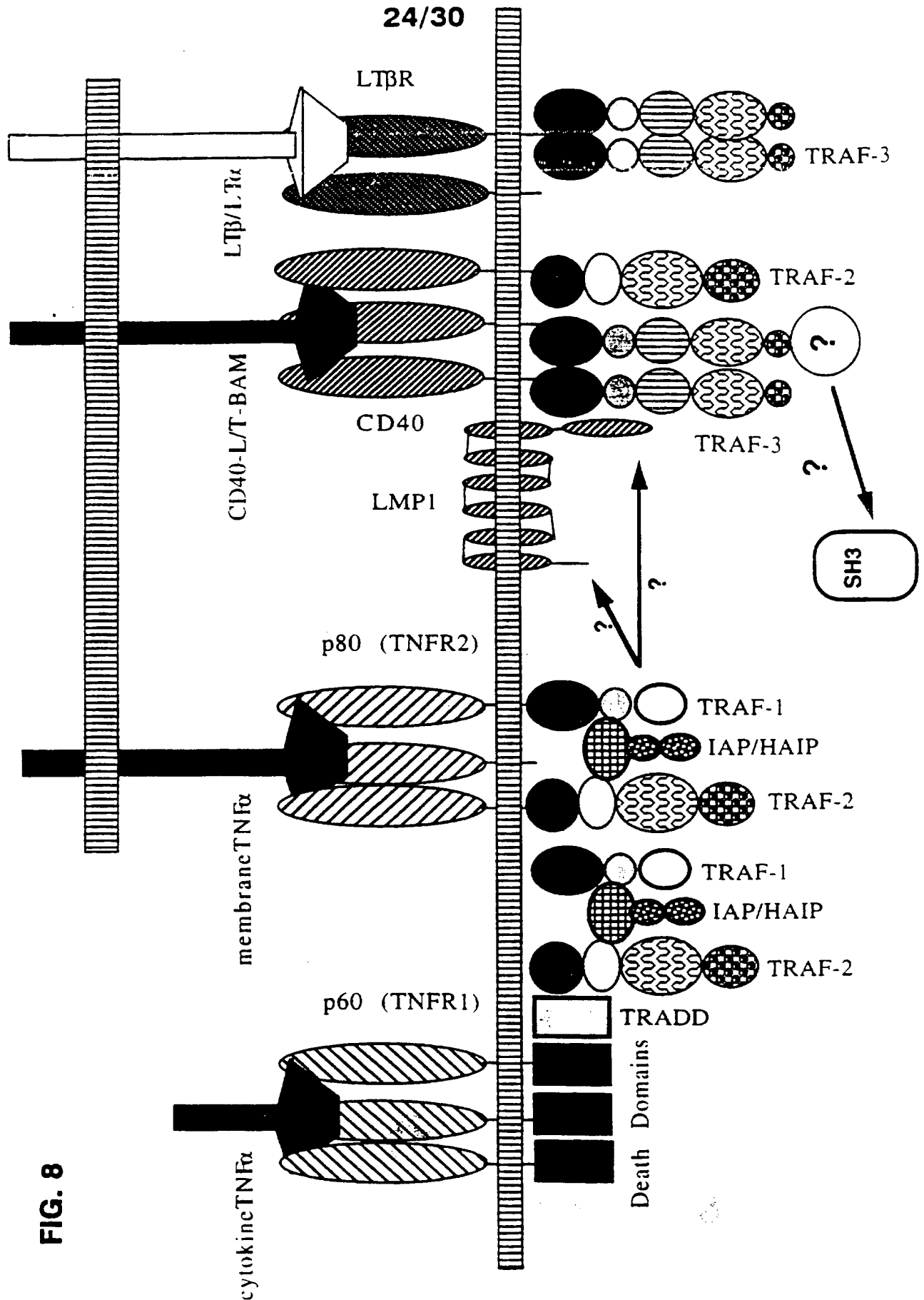
FIG. 7A



23/30

FIG. 7B



**FIG. 8**

SUBSTITUTE SHEET (RULE 26)

25/30

FIG. 9A

TRAF-3 p55

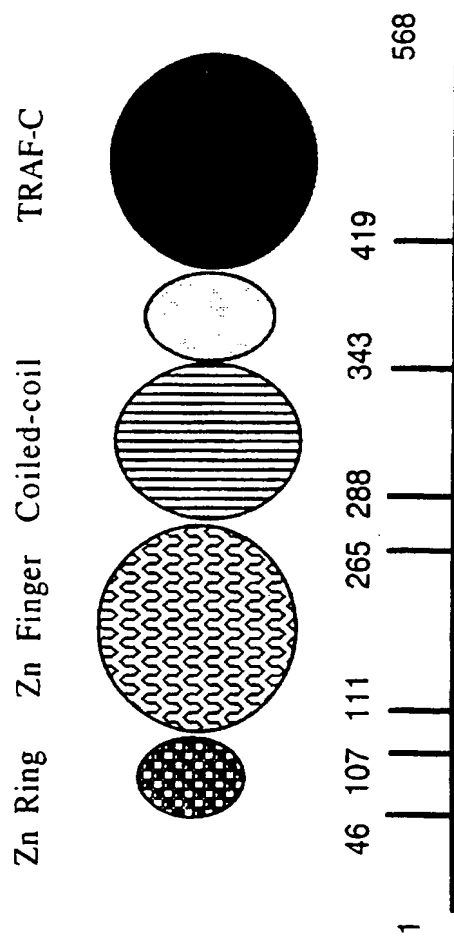
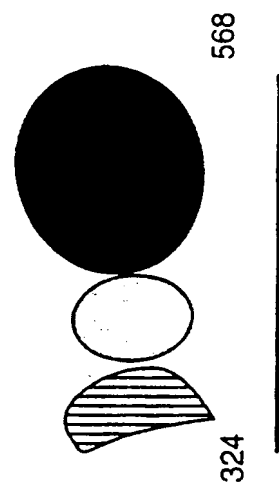


FIG. 9B

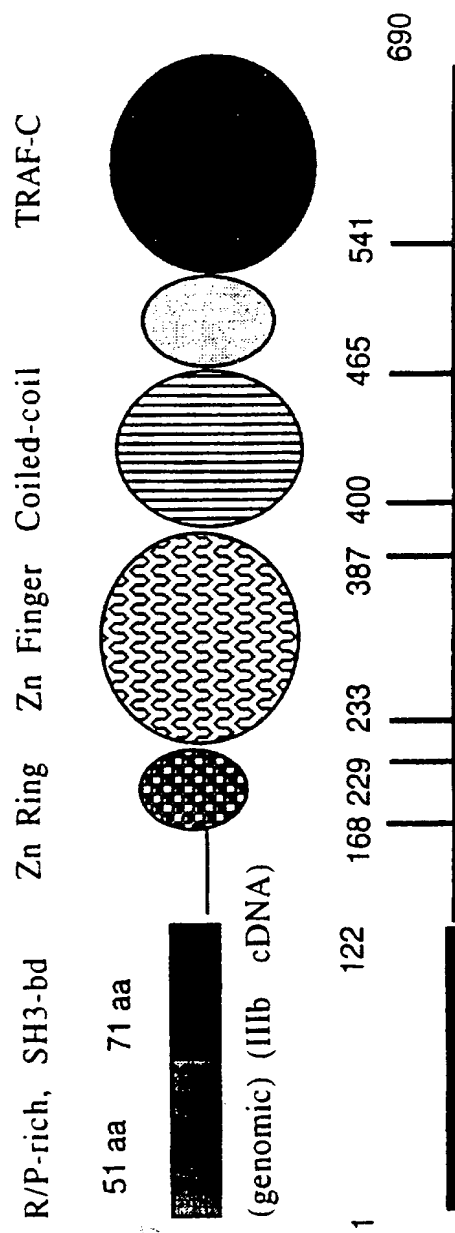
C26-Dominant Negative



26/30

FIG. 9C

TRAF-3 p70



27/30

FIG. 10A

1 TGT TTT NGT AAN AGG TTCA CAT NTGCC GGTGTTGA ATCTACT NNGNA ATTGC NNCCGGG
-----+-----+-----+-----+-----+-----+ 60
ACAAA NCATNTTCCA AGTGTANACGGCCACA AACTTAGATGANN CNNTTAACGNNGGCCCC
_residue -856 relative to cDNA map (putative transcription
initiation
61 CCTCCAAA TNNTGAA TAAAGTGGACCA CAGCCNGTCATTTCTGTNTTTAAANAACCAA
-----+-----+-----+-----+-----+-----+ 120
GGAGGTTTANNACTTATTCACCTGGTGGTCTGGNCAGTAAAGACAANA AATTNTTGGTT
121 AATATTCA NCTTGAAGGGGNTTTCTGNTTTGGCAAATTAAACCAGCCATAGTAAACA
-----+-----+-----+-----+-----+-----+ 180
TTATAAGTNGAACCTTCCCCANAAGACNA AACCGTTTAATTGGTGGTATCATTTTGT
181 CAGCATAAGCCAGCGTTGGATGGCCCCANTCAACAGGTGCCAGGANTGTGGNTAAGCAGG
-----+-----+-----+-----+-----+-----+ 240
GTCGTATTCGGTCGCAACCTACCGGGGTNAGTTGTCCACGGTCCCTNACACCNATTCTGTCC
241 CTGGGAACATGGAGCCTCAGTGCTTGGNTATGGGAAGTGTGGACCCCTGAGCCCTGAGTTC
-----+-----+-----+-----+-----+-----+ 300
GACCCCTGTACCTCGGAGTCACGAACCNATACCCCTTCAACAACCTGGGACTCGGACTCAAG
301 AGTAACATCCCAANTCCTGACAGGCAAGAAAGGCACCTGAGGNTCCCAAGGGANTCCACC
-----+-----+-----+-----+-----+-----+ 360
TCATTGTAGGGTTNAGGACTGTCCGTTCTTCCGTGGACTCCNAGGGTTCCCTNAGGTGG

28/30

FIG. 10B

```
ATGGGGTGGGGAGACCGCTGGAGTCCATAGGTCCCTGTGAGGGACGGCGAAGCGGGC 420
-----+-----+-----+-----+-----+-----+
361 TACCCCAACCCCTCTGGGACCTCAGGTATCCAGGGACACTCCCTGCCGCTTCCGCCCG

CTGTCACCTCTAAAAGAGCTGTGCCTGTGGCGCAGACGGGGGTGCCACTGTGGCCTGGTG 480
-----+-----+-----+-----+-----+-----+
421 GACAGTGGAGATTCTCGACACGGACACCGGCTCTGCCCCCAGGGTGACACCGGACCAC

AGTGTGGCTGTAGAACAAGCATCTGTGGGCTAATGGAGCCAGAGACATGGTCCAATGTC 540
-----+-----+-----+-----+-----+-----+
481 TCACACCGACATCTGTTCGTAGACACCCGATTACCTCGGTCTCTGTACCAGGTTACAG

TCTGATGTCCAGATAGACTTCCAGCAGCCAGGTCGGACTATCTGGGGTNTGGAACGTTTC 600
-----+-----+-----+-----+-----+-----+
541 AGACTACAGGTCTATCTGAAGGTCGTCCGGTCCAGCCTGATAGACCCCAANACCTTGCAAG
```

29/30

FIG. 10C

```

601 CATGGGTGAAGCTGTAGGAGGAGATGCTGGACTTCCTGCCCTCCACTCACATGAGTCACTG
-----+-----+-----+-----+-----+ 660
GTACCCACTTCGACATCCTCCTCTACGACCTGAAGGACGGAGGTGAGTGTACTCAGTGAC
putative AP-1 site

661 GAGCAATACAAGAAGGCAGACTGCCCTTGGCTACTCACACTGGTCTCAGCTCTGGGGTAG
-----+-----+-----+-----+-----+ 720
CTCGTTATGTTCTTCCGCTGTGACCGGAACCGATGAGTGTGACCAAGTCTCGAGACCCCATC
putative "CCAAT" box

*
721 GGCTCTTTATAAGCCCTTCTTGAGAAGTGAAAATGCAAAGTCTTCTGTCTCCTCACCA
-----+-----+-----+-----+-----+ 780
CCGAGAAATATTCGGAAGAACTCTTCACCTTTTACGTTTCAGGAAGACAGAGAGGTGGT
putative "TATA" box
putative cap-site
(transcription initiation site
termed nt 1 in Figure 1)

781 CTGACTTGCAAACTCCAGCGAAGGCCCTCACCCCTCAGCTGGCCAGAGCCTCCAGAAGC
-----+-----+-----+-----+-----+ 840
GACTGAACGTTTGAGGTCGCTTCGGAGTGGGGAGTCGACCGGGTCTCGGAGGGTCTTCG

```

30/30

FIG. 10D

841 CCTCCCTCGCTTCCACCTCGCCAGGCCAGCCAGGGTGGTGCTGCTTGGTGGGCCT + 900
GGAGGGAAGCGAAGGGTGGAGCGGTCCGGGTCCGCTCCACCCACGACGGAACCCCGGA

901 AGGTGAATCACTGACACACCACTGATGCTTTGGGGCAGAAAGCAGAGGCCTCGGGAGAA + 960
TCCACTTAGTGACTGTGTGGTGACTACGGAAACCCCGTCTTCCGTCTCCGAGGCCCTCTT

M P L G Q K A E A S G E -
_start of p70
_nt residue 169 of cDNA map

961 ATGCGCAGGCCCCAGCGGCCCTGGTGGAGGGTGCAGGGGG + 999
TAGCGTCCGGGGTCGCCGGACCACTCCACGTCCTCCCC

M R R P Q R P G G G C R G -

a

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/05076

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 435/2, 7.1; 424/93.21, 185.1, 534; 514/12, 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/2, 7.1; 424/93.21, 185.1, 534; 514/12, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CAPLUS, WPIDS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TEWARI et al. Recent Advances in Tumor Necrosis Factor and CD40 Signaling. Current Opinion in Genetics and Development. February 1996, Vol. 6, pages 39-44, see entire document.	1-87
Y	WARE et al. Apoptosis Mediated by the TNF-Related Cytokine and Receptor Families. J. Cell. Biochem. January 1996, Vol. 60, pages 47-55, see entire document.	1-87
Y	MORIO et al. Characterization of a 23-kDa Protein Associated with CD40. Proc. Natl. Acad. Sci. USA. December 1995, Vol. 92, pages 11633-11636, see entire document.	1-87

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance		
"E" earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search

23 MAY 1997

Date of mailing of the international search report

26 JUN 1997

Name and mailing address of the ISA/US,
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

EVELYN M. RABIN

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US97/05076

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROTHE et al. TRAF2-Mediated Activation of NF- κ B by TNF Receptor 2 and CD40. Science. 08 September 1995, Vol. 269, pages 1424-1427, see entire document.	1-87
Y	CHENG et al. Involvement of CRAF1, a Relative of TRAF, in CD40 Signaling. Science. 10 March 1995, Vol. 267, pages 1494-1498, see entire document.	1-87
Y	SATO et al. A Novel Member of the TRAF Family of Putative Signal Transducing Proteins Binds to the Cytosolic Domain of CD40.- FEBS Letters. January 1995, Vol. 358, pages 113-118, see entire document.	1-87
Y	HU et al. A Novel RING Finger Protein Interacts with the Cytoplasmic Domain of CD40. J. Biol. Chem. December 1994, Vol. 269, pages 30069-30072, see entire document.	1-87
Y	GORDON, J. CD40 and Its Ligand: Central Players in B Lymphocyte Survival, Growth, and Differentiation. Blood Reviews. March 1995, Vol. 9, pages 53-56, see entire document.	1-87
Y	BANCHEREAU et al. The CD40 Antigen and Its Ligand. Annu. Rev. Immunol. 1994, Vol. 12, pages 881-922, see entire document.	1-87
Y	FOY et al. The Expansive Role of CD40 and Its Ligand, gp39, in Immunity. Seminars in Immunology. October 1994, Vol. 6, pages 259-266, see entire document.	1-87
Y	DURIE et al. The Role of CD40 in the Regulation of Humoral and Cell-mediated Immunity. Immunology Today. September 1994, Vol. 15, No. 9, pages 406-411, see entire document.	1-87
Y	GRUSS et al. Tumor Necrosis Factor Ligand Superfamily: Involvement in the Pathology of Malignant Lymphomas. Blood. June 1995, Vol. 85, No. 10, pages 3378-3404, see entire document.	1-87
Y	US 5,434,248 A (CHAPMAN et al.) 18 July 1995, see entire document.	1-87
Y	US 5,177,085 A (R. NAEF) 05 January 1993, see entire document.	1-87
Y, P	US 5,518,729 A (S. MARGOLIN) 21 May 1996, see entire document.	1-87

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/05076

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A01N 1/02, 43/04, 63/00, 65/00; G01N 33/53; A61K 31/70, 38/00, 39/00, 35/14;